Mesangial cells initiate compensatory renal tubular hypertrophy via IL-10-induced TGF-β secretion: effect of the immunomodulator AS101 on this process

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Sinuani, Inna, Zhan Averbukh, Inna Gitelman, Micha J. Rapoport, Judit Sandbank, Michael Albeck, Benjamin Sredni, and Joshua Weissgarten. Mesangial cells initiate compensatory renal tubular hypertrophy via IL-10-induced TGF-β secretion: effect of the immunomodulator AS101 on this process. Am J Physiol Renal Physiol 291: F384–F394, 2006. First published March 28, 2006; doi:10.1152/ajprenal.00418.2005.—The present study investigated the role of IL-10 produced by the mesangial cells in postnephrectomy compensatory renal growth and the effect of the immunomodulator AS101 on this process. One hundred forty unilateral nephrectomized and sham-operated male Sprague-Dawley rats were treated by AS101 or PBS before and after surgery. The results show that secretion of IL-10 and TGF-β by mesangial cells isolated from the remaining kidneys was increased significantly, compared with those of control and sham animals. Moreover, TGF-β secretion by mesangial cells was increased after the addition of exogenous recombinant IL-10 and inhibited in the presence of neutralizing anti-IL-10 antibodies. In vivo, compensatory growth of the remaining kidneys was associated with significant increase in IL-10 content in renal tissues and plasma. Immunohistochemical studies show that IL-10 was produced by mesangial cells. Elevated IL-10 levels were followed by the rise in TGF-β content in plasma and renal tissue. AS101 treatment decreased IL-10 and TGF-β expression in plasma and kidney tissues and results in 25% reduction in the fresh and fractional kidney weight and decreased hypertrophy of tubular cells (protein/DNA ratio, morphometric analysis). Taken together, these data demonstrate that TGF-β production by mesangial cells is IL-10 dependent. Mesangial cells are the major source of IL-10 in kidneys. AS101, by inhibiting the activity of IL-10, decreases TGF-β production by mesangial cells, thus limiting compensatory tubular cell hypertrophy.

unilateral nephrectomy; cytokines; tubular cells

UNILATERAL NEPHRECTOMY (Nx) results in compensatory renal growth (CRG), in which both the size and the functional capacity of the remaining kidney are increased. Loss of “working” nephrons through the removal of the contralateral kidney leads to functional adaptation of the remaining nephrons. This adaptation is characterized by an increase in the glomerular filtration rate (GFR) and hypertrophy of cells comprising the nephron, which is primarily of proximal tubular origin (8, 55). Increased synthesis and production of local growth factors after nephron reduction are an essential factor in triggering compensatory growth of the remaining nephrons (9, 36).

Transforming growth factor (TGF)-β is a multifunctional growth factor that participates in the regulation of cell proliferation, accumulation of extracellular matrix, glomerular and interstitial fibrosis, and the progression of glomerulosclerosis (28, 48). It has been implicated as one of the most important factors causing tubular cell hypertrophy (5, 57). TGF-β is therefore considered to have a pivotal role in CRG (11).

Glomerular mesangial cells, which participate in most physiological and pathological renal processes, secrete and respond to a variety of growth factors, cytokines, and chemokines, such as EGF, ANG II, endothelin, IGF, IL-6, IL-1, IL-10, TNF-α, and others (2, 10, 39, 46). Moreover, among the resident renal cells studied, only mesangial cells have been found to secrete and activate TGF-β (22, 23).

The anti-inflammatory Th2 cytokine IL-10 first recognized for its ability to inhibit activation and effectors 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 is to limit and ultimately terminate inflammatory responses. IL-10 regulates growth and/or differentiation of B cells, NK cells, cytotoxic and T helper cells, mast cells, keratinocytes, and endothelial cells (29). Moreover, IL-10 was recently reported to be an autocrine growth factor affecting mesangial cells. Indeed, IL-10 induces a dose-dependent proliferation of growth-arrested mesangial cells in vitro. IL-10 administration to normal rats in vivo results in an increased number of glomerular cells and transient reduction of creatinine clearance (4). Furthermore, IL-10 gene and protein expression have also been observed within glomeruli from biopsies obtained from patients with IgA nephropathy, suggesting a role in human mesangioproliferative glomerulonephritis (30). Studies have demonstrated an association between the pathophysiology of various kidney diseases, all of which are related to mesangial cell proliferation, such as mesangioproliferative glomerulonephritis, IgA nephropathy, and the acute phase of microscopic polyangiitis, and upregulation of IL-10 (21, 38, 60). Moreover, IL-10 can promote mesangial deposition of the immune complex, and thus contribute to the progression of glomerular injury (24).

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It has been shown that IL-10 and TGF-β act synergistically to regulate production of proinflammatory cytokines, nitric oxide, and others by mononuclear cells (3, 16, 31, 61) and that TGF-β enhances IL-10 mRNA and protein expression in various cell types, including mesangial cells (2, 27). Moreover, in the model of experimental colitis, Fuss et al. (12) show that IL-10 is a necessary factor to facilitate TGF-β production by CD4+ T cells.

Several preclinical and clinical studies demonstrated a beneficial effect of the nontoxic immunomodulator AS101 [ammonium trichloro (dioxoethylene-O,O')tellurate] on adverse events associated with overproduction of IL-10 (17, 44). AS101 blocks the transcription of IL-10 mRNA without affecting TNF-α and IL-1 mRNA levels (47). A direct inhibition of IL-10 at the level of mRNA apparently underlies most of AS101’s immunomodulatory activity. This is followed by increase of certain cytokines such as IL-1α, TNF-α, interferon-γ, IL-2, and a colony-stimulated growth factor (43, 44). Furthermore, in a murine model of septic peritonitis, AS101 was recently shown to prevent kidney damage of septice mice (19). In addition, AS101 was recently shown to decrease the spontaneous production of IL-10 by peripheral blood mononuclear cells (PBMC), both in vitro and in vivo, in patients with systemic lupus erythematosus (SLE). Moreover, AS101 treatment of NZB/WF1 mice that spontaneously develop SLE, or of SCID mice injected with PBMC from human SLE patients, delays the appearance of autoimmune manifestations. These benefits of AS101 include reduced immune complex deposition in the glomeruli, prevention of glomerular hypercellularity and mesangial expansion, and decreased proteinuria (18). AS101 administration to rats with Thy1-induced glomerulonephritis extensively decreased glomerular mesangial cell expansion and protein excretion (20). In addition, AS101 through its inhibitory effect on IL-10 negative regulates expression of GDNF, an autocrine mesangial cells growth factor. This growth factor plays an important role in the pathogenesis of rat mesangioproliferative glomerulonephritis (21).

The aims of the present study were to investigate 1) whether mesangial cells have a role in compensatory renal growth through IL-10 secretion, 2) the possibility of interrelationships between IL-10 and TGF-β production by the mesangial cells, and 3) the influence of inhibition of IL-10 and TGF-β by the immunomodulator AS101 on the development of compensatory tubular cell hypertrophy of the remaining kidneys.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, each 8 wk old and weighing ~180 g, were purchased from Harlan Laboratories. All experiments were conducted according to National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental design. Rats underwent either right nephrectomy or sham nephrectomy (sham) under 1.5% halothane anesthesia (Rhodia, Bristol, UK). A total of 140 rats were used for in vitro and in vivo studies. In the in vitro studies, mesangial cells were separated from 5 control rats, which did not undergo nephrectomy, 5 sham, and 10 Nx rats.

In the in vivo studies, a total of 120 rats were divided in four groups: sham rats (n = 30) injected intraperitoneally with PBS (0.5–1.2 ml according to weight); sham rats (n = 30) injected intraperitoneally with AS101 (0.5 mg/kg); Nx rats (n = 30) injected intraperitoneally with PBS (0.5–1.2 ml according to weight); and Nx rats (n = 30) injected intraperitoneally AS101 (0.5 mg/kg).

All rats were treated by either AS101 or PBS once on alternate days during the week before surgery and 4 wk after surgery. Rats were anesthetized, weighed, and blood samples were taken from the heart. After these samples were drawn, the rats were immediately killed. Rats from each group (n = 5) were killed at 24, 48, and 72 h, 1, 2, and 4 wk after unilateral/sham nephrectomy. The remaining kidneys of Nx rats and the left kidneys of sham rats were removed.

The following studies were performed: 1) blood count, 2) concentrations of IL-10 and TGF-β in plasma of each experimental animal were quantified by ELISA, 3) expression of IL-10 and TGF-β in the renal cortices were assessed by Western blot analysis, 4) immunohistochemical double staining for IL-10 and Thy1.1, 5) fractional kidney weight (FKW) ratio was expressed as the ratio between the fresh kidney weight and the body weight × 100%; 6) assessment of protein/DNA ratio; and 7) morphometric analyzes.

AS101. The immunomodulator AS101 was synthesized in the Department of Chemistry, Bar-Ilan University and was supplied as a sterile solution at a concentration of 1.5 mg/100 ml maintained at 4°C. Before use, the AS101 stock was diluted in PBS (pH 7.4) to achieve the required concentrations.

Separation and culture of mesangial cells. Mesangial cells were separated from kidneys of control (nonoperated), sham, and Nx rats, using a method described by Averbukh et al. (1). The removed kidneys were cleaned of fat, encapsulated, and washed several times in PBS (pH 7.4). Cortices were then separated from medullas, cut into small pieces, incubated with 0.1% collagenase for 20 min, passed serially through 200- and 90-μm stainless steel meshes, and washed three times with PBS. Cell clusters were resuspended and grown in 24-well plates in RPMI-1640 containing d-valine instead of l-valine and supplemented with 20% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml neomycin (Biological Industries, Kibbutz Beit Ha-Emek, Israel) at 37°C, 5% CO2, 95% humidity.

Cell cultures were examined serially under a phase-contrast light microscopy during the growth period. These cells had the typical appearance of spindle-shaped bundles, and no polygonal endothelial or epithelial cells could be detected. Cells were stained positive with a rabbit polyclonal anti-rat Thy-1.1 antibody (mesangial cell marker; Zymed Laboratories). Staining with ED-1 monoclonal antibody to rat macrophages was negative. Contamination by fibroblasts was excluded by culturing mesangial cells in media in which d-valine replaces the natural L-isomer, thus blocking the growth of most fibroblast cell lines in vitro due to a deficiency in d-amino acid oxidase (25). The total protein content of cells collected from each well was detected by DC protein assay (Bio-Rad Laboratories, Richmond, VA).

Reach confluent at passages 2–4 were used for in vitro studies. For the ELISA assay, mesangial cells were replaced in 24-well plates. Before stimulation, cells were starved for 24 h with RPMI-1640 containing d-valine supplemented with 0.1% FCS and 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml neomycin.

Evaluation of IL-10 and TGF-β production by cultured mesangial cells. Mesangial cells isolated from the kidneys of control, sham, and Nx rats were stimulated with/without 10 μg/ml LPS derived from Escherichia coli 026B6 (Sigma) to increase IL-10 production by them, and/or with AS101, recombinant rat IL-10, neutralizing anti-IL-10 Ab (R&D Systems) at different concentrations. Following stimulation, the supernatants were collected. Cells from each well were scraped, lysed, and protein content was measured. Concentrations of IL-10 and TGF-β in the plasma and mesangial cell supernatants were estimated using a cytokine-specific ELISA kits (Endogen, Boston, MA). To assess the total TGF-β levels in the mesangial cell supernatants, all of TGF-β sample content was converted to the active forms, according to the manufacturer’s protocol (R&D Systems). Cytokine production by mesangial cells was calculated as cytokine concentration in the supernatant per 10 μg of protein content.
**Plasma procurement.** Control, sham, or Nx rats were anesthetized using 1.5% halothane anesthesia. Blood samples were taken from the heart using a single-use syringe washed with 5,000 U/ml heparin sodium. All rats were then immediately killed. Blood samples were replaced in 15-ml sterile test tubes and centrifuged for 15 min at 3,000 rpm. Plasma sample from each animal was passed through 0.2-μm syringe filter (Millipore Israel). Plasma samples were stored at −20°C.

**Western blot analysis.** Kidney cortices were separated from medulla, cut into small pieces, passed through a 200-μm stainless steel mesh, and washed twice with ice-cold PBS (pH 7.4). After being subjected to hypotonic shock, cell lysates were prepared by incubation for 20 min on ice with 200 μL of ice-cold lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.2, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate, 10% glycerol, 30 mM NaF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin; Sigma, St. Louis, MO). These lysates were then centrifuged at 4°C for 10 min at 14,000 rpm. Protein concentration in the lysates was measured by DC-protein assay (Bio-Rad). For electrophoresis, equal concentrations of proteins from each kidney were loaded per lane and separated on 10% Tris-HCl ready gels (Bio-Rad). Proteins were transferred to nitrocellulose membranes and probed with appropriate antibodies. Proteins were visualized by ECL according to the instructions of the manufacturer (Amersham, Buckinghamshire, UK). Nonspecific binding was blocked by incubation for 1 h with a blocking buffer (5% low-fat milk, 5% fetal calf serum, 10 mM Tris, pH 7.5, 0.1% Tween 20, 100 mM NaCl; Sigma).

**Immunohistochemistry.** Kidney samples were dissected, immediately embedded in OCT (Tissuetek), oriented, and frozen in liquid N2. Sections (10–12 μm) were collected on Gold+ slides (Menzel-Gläser) in a Leica cryostat microtome, air dried at room temperature for 1 h, and processed for immunohistochemistry. The dried sections were fixed for 5 min at room temperature in freshly prepared 4% paraformaldehyde (PFA) in PBS, washed with TBS, and several changes of PBS. Sections then were incubated with a rabbit polyclonal anti-rat Thy-1.1 antibodies (1:50) for 1.5 h, washed three times with TBS, and incubated with rabbit anti-mouse IgG TRITC-conjugated secondary antibodies (1:50) for 30 min in dark, and washed three times with PBS. Immediately after PBS washes, sections were incubated with goat anti-ready anti-IL-10 antibody (R&D Systems; 1:10) for 3 h at dark place, washed with PBS, and incubated with rabbit anti-goat IgG FITC-conjugated antibodies (Zymed Lab; 1:25) for an additional 30 min, after which the three 10-min PBS washes were repeated. The secondary antibodies were diluted in PBS containing 10% normal rabbit serum to reduce nonspecific Fc-binding. The slides were then mounted with mounting medium for fluorescence containing DAPI (Zymed Lab).

**Image processing.** Staining was visualized using a Olympus BX51 fluorescent microscope and a rhodamine, FITC, and DAPI filter set. The images were collected with the digital cooled CCD SPOT camera (RT Slider SPOT, Diagnostic Instruments), processed with Photoshop 7.0 (Adobe), and printed on a dye sublimation printer (Kodak).

**DNA determination.** Kidneys were pulverized and kept frozen in liquid nitrogen until analysis. DNA was extracted using Tri Reagent (Sigma) according to the manufacturer’s protocol. In brief, 1 ml of Tri Reagent was added to 70–100 mg renal tissues and centrifuged at 2,000 g for 15 min at 4°C. DNA was isolated from the middle phase and phenol phase separated from the initial homogenate by addition of 100% ethanol for 2–3 min at room temperature and following centrifugation at 2,000 g for 5 min at 4°C. The DNA pellet was washed twice in 0.1 M sodium citrate in 10% ethanol, dried for 10 min under vacuum, and dissolved in 8 mM NaOH. Concentrations of DNA were quantities by spectrophotometer at 260 and 280 nm. The amounts of DNA are free of RNA and proteins have a 260/280 ratio >1.7. Aliquots of the same homogenates were used to determine protein concentration by DC-protein assay (Bio-Rad Lab).

**Morphometric analysis.** Tissues were fixed in 4% buffered formalin and embedded in paraffin. Sections (3-μm thick) were stained with hematoxylin and eosin. Morphometric analyses were performed with a computerized microscopy using the Image Pro Media (CyberMetrics) program. Sections from each kidney were evaluated by two independent observers blinded to the treatment administrated. From each kidney, 50 glomeruli, 50 sections of proximal tubular cells, and 50 sections of distal tubular cells were scanned to assess the following parameters: mean number of cellular nuclei per glomerulus (GC), mean glomerular area (GA), mean proximal tubular cell area (PTCA), and mean distal tubular cell area (DTCA).

**Statistical analysis.** Statistical analyses were performed using the ANOVA with Bonferroni correction for multiple comparisons. Values are expressed as means ± SD. Correlation was calculated using Pearson’s correlation test. A value of P < 0.05 was considered statistically significant.

**RESULTS**

**IL-10 and TGF-β production by cultured mesangial cells.** IL-10 and TGF-β concentrations in the mesangial cell supernatants were significantly higher following stimulation with 10 μg/ml LPS compared with the basal levels (Fig. 1). Production of IL-10 and TGF-β by nonstimulated and LPS-stimulated cultured mesangial cells derived from the remaining kidneys increased significantly, compared with those obtained from the kidneys of sham and control rats. Moreover, the amounts of IL-10 and TGF-β secreted by mesangial cells were positively correlated; R = 0.989 for mesangial cells from normal kidneys; R = 0.983 for mesangial cells from sham kidneys; and R = 0.999 for mesangial cells from the single remaining kidneys. These data indicate that basal and LPS-stimulated secretion of IL-10 and TGF-β by mesangial cells is upregulated following unilateral nephrectomy. It is also demonstrated that sham operation by itself enhances the production of these cytokines.

**Effect of IL-10 on TGF-β production by mesangial cells.** To examine the effect of IL-10 on TGF-β production, mesangial cells were isolated from control (nonoperated) rats and were stimulated with LPS in the presence of recombinant rat IL-10 or neutralizing anti-IL-10 antibodies. The addition of rat IL-10 to LPS-stimulated cultures of mesangial cells increased TGF-β production in a dose-dependent manner (Fig. 2A). IL-10 at 100 ng/ml increased TGF-β production about twofold (from 1,471 ± 127 to 2,539 ± 264 pg/10 μg protein, P < 0.001) and was found to be the most effective. Furthermore, TGF-β production by LPS-stimulated mesangial cells was reduced almost to the basal levels (486 ± 58 pg/10 μg protein) when neutralizing anti-IL-10 antibodies at 0.1 and 1 μg/ml concentrations were added to the cultures (Fig. 2B). These results indicate that IL-10 regulates the production of TGF-β.

**Effect of AS101 on IL-10 production by cultured mesangial cells.** To examine the influence of AS101 on IL-10 production, mesangial cells were isolated from kidneys of control, sham, and Nx rats. Mesangial cells were either nonstimulated or stimulated with LPS at 10 or 20, 100 mM NaCl; Sigma).

**Immunohistochemistry.** Kidney samples were dissected, immediately embedded in OCT (Tissuetek), oriented, and frozen in liquid N2. Sections (10–12 μm) were collected on Gold+ slides (Menzel-Gläser) in a Leica cryostat microtome, air dried at room temperature for 1 h, and processed for immunohistochemistry. The dried sections were fixed for 5 min at room temperature in freshly prepared 4% paraformaldehyde (PFA) in PBS, washed with TBS, and several changes of PBS. Sections then were incubated with a rabbit polyclonal anti-rat Thy-1.1 antibodies (1:50) for 1.5 h, washed three times with TBS, and incubated with rabbit anti-mouse IgG TRITC-conjugated secondary antibodies (1:50) for 30 min in dark, and washed three times with PBS. Immediately after PBS washes, sections were incubated with goat anti-ready anti-IL-10 antibody (R&D Systems; 1:10) for 3 h at dark place, washed with PBS, and incubated with rabbit anti-goat IgG FITC-conjugated antibodies (Zymed Lab; 1:25) for an additional 30 min, after which the three 10-min PBS washes were repeated. The secondary antibodies were diluted in PBS containing 10% normal rabbit serum to reduce nonspecific Fc-binding. The slides were then mounted with mounting medium for fluorescence containing DAPI (Zymed Lab).

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**DNA determination.** Kidneys were pulverized and kept frozen in liquid nitrogen until analysis. DNA was extracted using Tri Reagent (Sigma) according to the manufacturer’s protocol. In brief, 1 ml of Tri Reagent was added to 70–100 mg renal tissues and centrifuged at 2,000 g for 15 min at 4°C. DNA was isolated from the middle phase and phenol phase separated from the initial homogenate by addition of 100% ethanol for 2–3 min at room temperature and following centrifugation at 2,000 g for 5 min at 4°C. The DNA pellet was washed twice in 0.1 M sodium citrate in 10% ethanol, dried for 10 min under vacuum, and dissolved in 8 mM NaOH. Concentrations of DNA were quantities by spectrophotometer at 260 and 280 nm. The amounts of DNA are free of RNA and proteins have a 260/280 ratio >1.7. Aliquots of the same homogenates were used to determine protein concentration by DC-protein assay (Bio-Rad Lab).

**Morphometric analysis.** Tissues were fixed in 4% buffered formalin and embedded in paraffin. Sections (3-μm thick) were stained with...
Nx induces a transient marked elevation in IL-10 plasma content and this increase was downregulated by AS101.

**Plasma TGF-β levels.** TGF-β plasma concentrations (Fig. 6) were reduced 24 h after unilateral nephrectomy (29 ± 4 ng/ml), compared with those in nonoperated rats (40 ± 4 ng/ml, **P** < 0.05). Forty-eight hours following unilateral nephrectomy, TGF-β levels gradually increased (44 ± 9 ng/ml at 48 h, 49 ± 4 ng/ml at 72 h) and peaked at 1 wk postnephrectomy (56 ± 4 pg/ml, **P** < 0.05). Following this period, the levels of TGF-β gradually returned to normal. AS101 treatment reduced significantly the levels of TGF-β from 24 h to 1 wk. TGF-β levels in the plasma of sham rats treated or untreated with AS101 did not change significantly, compared with normal levels in each time period. Thus Nx resulted in temporary increase in TGF-β plasma levels and this increase occurred sequentially to the rise in IL-10 levels.

**IL-10 expression in renal tissues.** IL-10 expression in the remaining kidney cortices razed up within 24 h following unilateral nephrectomy (Fig. 7) and returned to basal levels following 1 wk nephrectomy. A second and smaller elevation of IL-10 at 2 wk after nephrectomy was detected. Treatment with AS101 significantly reduced IL-10 at 24 – 72 h in Nx and 2 wk. These changes in IL-10 tissue content were not observed in sham rats untreated and treated with AS101. Thus these data show that IL-10 expression in the renal tissues peak at 24 h postnephrectomy in parallel to the rise of plasma levels.

Incubation of LPS-stimulated mesangial cells with AS101 resulted in about two- to threefold reduction in the amounts of TGF-β. The addition of 100 ng/ml of recombinant rat IL-10 to the cultures neutralized the effect of AS101 on TGF-β secretion. Thus AS101 inhibits TGF-β secretion by mesangial cells through its inhibitory effect on IL-10.

**Plasma IL-10 levels.** Figure 5 demonstrates that 24 h following operation, the IL-10 plasma levels of Nx rats were significantly higher than in sham rats (214 ± 65 vs. 48 ± 18 pg/ml, respectively; **P** < 0.001). From 48 h after unilateral nephrectomy, IL-10 levels gradually decreased to the basal value and remained constant for 4 wk.

AS101 treatment decreased high IL-10 plasma concentrations in Nx rats but did not affect normal IL-10 levels. In sham rats, slight elevation of IL-10 content in plasma after 24 h (to 48 ± 18 pg/ml, **P** < 0.05) was found, and, at this time, AS101 decreased IL-10 content in plasma to 22 ± 16 pg/ml. From 48 h to 4 wk, IL-10 concentrations in the plasma of sham rats were normal and unchanged following AS101 treatment. Thus
To clarify the exact source of renal IL-10 production, we examine the cryosections from kidneys of sham rats and a remaining kidneys from Nx rats (Fig. 8). IL-10 expression was observed only in the intraglomerular mesangial cell areas, but not in tubular cells. IL-10 expression (upper line) in the kidneys of Nx rats treated by PBS was remarkably higher than in sham and AS101-treated Nx rats. As can be seen in the merged image (bottom line) of double staining with anti-IL-10 and anti-Thy1.1 antibodies (mesangial cell marker), the expression of IL-10 in the remaining kidneys of PBS-treated rats was prominently confluent in the mesangial cell areas. Following AS101 treatment, the staining of IL-10 was significantly lower and visualized in the merged image as granular patches. This suggests that AS101 inhibits significantly IL-10 production by mesangial cells. These results show that the major source of IL-10 secretion following Nx in the remaining kidneys is the mesangial cells.

TGF-β expression in the renal tissues. Figure 9 shows that TGF-β levels in the remaining kidneys were reduced within the first 24 h compared with sham rats. The rise in TGF-β expression in the remaining kidney tissues started at 72 h and peaked at 1 wk after surgery. This expression was followed by a reduction to the basal levels after 2 to 4 wk. The elevated TGF-β expression was reduced by AS101, which did not affect normal TGF-β levels. Sham operation did not influence TGF-β expression in the kidneys of the PBS- or AS101-treated rats. Thus the expression of TGF-β in the renal tissues was correlated positively with plasma TGF-β levels.
Morphological and morphometric characteristics of the experimental kidneys. Table 1 depicts the morphometric data for the experimental kidneys. Two weeks after Nx, the mean fresh weight of the single kidneys from PBS-treated rats was twofold higher than the fresh weight of kidneys from sham rats (1,245 ± 76 compared with 640 ± 55 mg, \( P < 0.01 \)). The weight of single kidneys from AS101-treated rats was about 25% lower than the weight in PBS-injected Nx rats. The weight gain of the remaining kidneys after AS101 treatment was \( \Delta 456 \pm 18 \) mg, compared with \( \Delta 605 \pm 21 \) mg in PBS-treated rats.

The fractional kidney weight ratio (FKW) in PBS-injected rats 2 wk after Nx was significantly greater than in sham rats (0.51 ± 0.02 compared with 0.33 ± 0.02\%, \( P < 0.01 \)). After treatment with AS101, the FKW in the Nx rats (0.47 ± 0.02\%) was less than the weight in PBS-treated rats (\( P < 0.05 \)).

The protein/DNA ratio was increased significantly in the remaining kidneys, compared with sham. Furthermore, the protein/DNA ratio of kidneys from AS101-treated rats was reduced significantly compared with PBS-treated rats.

The mean glomerular surface area (GA) and the mean number of intraglomerular cells (GC) were unchanged after unilateral nephrectomy, but both GA and GC were significantly reduced by AS101 treatment. The mean proximal (PTCA) and distal (DTCA) tubular cell areas as expected increased after Nx. AS101 treatment resulted in a significant reduction in PTCA and DTCA compared with these cell areas in PBS-treated rats (\( P < 0.05 \)). Yet, AS101 treatment of sham rats had no effect on the morphological and morphometric parameters. Thus unilateral nephrectomy led to an increase in kidney weight, FKW ratio, protein/DNA ratio, and PTCA and DTCA, and these changes were partially inhibited by AS101 treatment.

**DISCUSSION**

The mechanisms of renal hypertrophy in response to loss of renal mass are fundamental to understanding the biology of the kidney and the progression of renal failure. TGF-\( \beta \) is known as one of the most important factors contributing to hypertrophy of renal tubular cells. The present study underscores the role of IL-10 in the initiation of TGF-\( \beta \) production by mesangial cells in CRG after Nx.

Our results show that concentrations of IL-10 in plasma and IL-10 expression in renal tissues were markedly increased 24 h after Nx. Moreover, the immunohistochemical studies revealed that in the remaining kidneys IL-10 was secreted primarily by the mesangial cells. To the best of our knowledge, this is the first report showing that mesangial cells are the major source of IL-10 in the remaining kidneys. Furthermore, our results demonstrate that cultured mesangial cells separated from the remaining kidneys 24 h after surgery produced elevated amounts of IL-10 with/without LPS stimulation, compared with cells obtained from kidneys of normal and sham-operated rats.
1). Differentiation of single kidney mesangial cells from their normal counterparts was manifested in cultures following passages 2-4 after the primary isolation. These cells thus retained in vitro several of features activated by a nonmutagenic manipulation, i.e., Nx, which had been performed in vivo. In fact, reports of a similar nature of this “memory” were previously published. For example, fibroblasts derived from fibrotic human lungs, following passages 4-8 in culture, retain a blunted capacity to produce collagen, compared with collagen production by fibroblasts isolated from normal lungs. (32). Moreover, conditioned medium from cultured lung fibroblasts isolated from patients with idiopathic pulmonary fibrosis or from par-

Fig. 8. Renal IL-10 distribution. Kidney samples were dissected, immediately embedded, and frozen in liquid N2. Sections (10–12 μm) were collected on Gold+ slides in a Leica cryostat microtome, air dried at RT for 1 h, and processed for immunohistochemistry. The cryosections from kidneys of sham rats and a remaining kidney from rats treated with PBS (Nx) and AS101 (Nx + AS101) were stained with anti-Thy1.1 (1:50) and anti-IL-10 (1:10) primary, and TRITS-conjugated and FITC-conjugated secondary antibodies. Photographs were prepared using Olympus BX51 fluorescent microscope in manual manner at the equal light power and exposure time conditions. The merged images of IL-10 + Thy1.1 were prepared using Photoshop 7.0 program. Magnification ×400. Inset: magnification ×100.
quet-treated rats was shown to induce exaggerated apoptosis of human A549 cell lines, or cultured from primary alveolar rat cells, respectively (34, 49). In addition, TGF-β-stimulated cultured myometrial cells obtained from the urethras of pregnant females produced exaggerated amounts of PTH-related protein, compared with uterine myometrial cells originating from nonpregnant women (37). Similarly pertinent data concerning cultured intraglomerular mesangial cells have been published from our laboratory. The data demonstrated that unilateral nephrectomy induces profound alterations in several of physiological responses of mesangial cells originated from the remaining kidneys. These alterations persist in long-term cultures. Thus mesangial cells harvested from a remaining kidney, but not from a control kidney, secrete a factor(s) which, in concert with serum procured from unilateral nephrectomized rats, stimulates cultured tubular cell proliferation (1). Cultured mesangial cells from a single kidney were found to release increased amounts of IL-6 in response to IL-1, compared with normal mesangial cell (52). In addition, the proliferate response of cultured mesangial cells obtained from the remaining kidneys to various growth factors was significantly decreased, compared with their normal counterparts (53). These observations demonstrate that upon exposure to an appropriate signal generated in vivo, mesangial cells as well as other cells acquire and retain in vitro the ability for an altered response to a given stimulus.

In vivo appearance of high IL-10 levels in plasma and increased IL-10 secretion by glomerular mesangial cells during first days following Nx suggests about role of this cytokine initiating CRG of the remaining kidneys. Our previous studies have suggested that mesangial cells are participating in the development of tubular cell hypertrophy in CRG (1, 52–54).

As known, TGF-β is considered as a pivotal factor in progression of renal hypertrophy. TGF-β is synthesized and secreted by a variety of cells composing the nephron (14, 22, 28). However, among the resident renal cells studied, only mesangial cells have been found to secrete TGF-β, in its latent form and convert it to its active state (22, 23).

IL-10 and TGF-β act synergistically in certain situations, such as macrophage deactivation, regulation of proinflammatory cytokines and nitric oxide production, and inhibition of RANTES production by microglial cells (3, 16, 31, 61). Our results show that increased production of IL-10 by cultured mesangial cells correlates positively with increased production of TGF-β. Moreover, we found that increased IL-10 and TGF-β expression in the plasma and renal tissue of unilateral nephrectomized rats associated with marked hypertrophy of proximal and distal tubular cells in vivo underscore their combined role in promoting compensatory growth of the remaining kidney.

However, IL-10 apparently has no significant effect on tubular cells (7, 13, 35). Moreover, in situ hybridization (30) and our immunohistochemical studies have both demonstrated that IL-10 does not stain positive in the tubular cell areas. TGF-β has been shown to induce the synthesis and production of IL-10 in various cell types, including mesangial cells (2, 27). The effect of IL-10 on TGF-β production has received sparse research attention (6, 50). It has been shown that in vivo administration of anti-IL-10 antibodies to mice with trinitrobenzene sulfonic acid (TNBS)-induced colitis prevented

Table 1. Morphological and morphometric parameters of kidneys 2 wk after unilateral/sham nephrectomy

<table>
<thead>
<tr>
<th></th>
<th>Sham + PBS (n = 5)</th>
<th>Sham + AS101 (n = 5)</th>
<th>Nx + PBS (n = 5)</th>
<th>Nx + AS101 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh kidney wt, mg</td>
<td>640±55</td>
<td>565±61</td>
<td>1,245±76*</td>
<td>1,094±69† (25)</td>
</tr>
<tr>
<td>Kidney wt/body wt, %</td>
<td>0.33±0.02</td>
<td>0.33±0.03</td>
<td>0.51±0.02*</td>
<td>0.47±0.02† (22)</td>
</tr>
<tr>
<td>Total renal DNA content, mg</td>
<td>6.34±0.24</td>
<td>6.29±0.29</td>
<td>6.46±0.37*</td>
<td>6.44±0.29*</td>
</tr>
<tr>
<td>Total renal protein content, mg</td>
<td>166.4±5.2</td>
<td>161.7±9.2</td>
<td>236.3±10.2*</td>
<td>200.3±12.1† (44)</td>
</tr>
<tr>
<td>Protein/DNA ratio</td>
<td>26.24±0.48</td>
<td>25.73±0.71</td>
<td>35.81±0.64*</td>
<td>33.35±0.64† (41)</td>
</tr>
<tr>
<td>Glomerular area, μm²</td>
<td>9,576±765</td>
<td>9,628±751</td>
<td>9,326±603</td>
<td>8,188±575† (12)</td>
</tr>
<tr>
<td>Number of intraglomerular cells</td>
<td>80±12</td>
<td>78±14</td>
<td>75±16</td>
<td>64±12† (15)</td>
</tr>
<tr>
<td>Proximal tubular cell area, μm²</td>
<td>262±30</td>
<td>268±32</td>
<td>374±60*</td>
<td>327±61† (47)</td>
</tr>
<tr>
<td>Distal tubular cell area, μm²</td>
<td>148±34</td>
<td>141±29</td>
<td>240±45*</td>
<td>189±42† (48)</td>
</tr>
</tbody>
</table>

Data are means ± SD. Percentage of decrease, differences between Δ₁ vs. Δ₂, is in parentheses; Δ₁ was assessed as differences between absolute levels of parameter of Nx-PBS-treated rats and Sham-PBS-treated rats. Δ₂ was assessed as differences between absolute levels of parameter of Nx-AS101-treated rats and Sham-AS101-treated rats. *P < 0.05 compared with sham rats. †P < 0.05 compared with sham and PBS-treated unilaterally nephrectomized rats.
TGF-β secretion by CD4+ T cells. It therefore demonstrated that IL-10 is a necessary factor to induce TGF-β production by CD4+ T cells (12). The results of this study show that stimulation of mesangial cells with exogenous rat IL-10 resulted in a significant increase in TGF-β production. Furthermore, inhibition of IL-10 by specific neutralizing antibodies abolished TGF-β production by LPS-stimulated mesangial cells (Figs. 2 and 4). Thus to the best of our knowledge, this study provides for the first time evidence of the cross talk between IL-10 and TGF-β production by mesangial cells, namely, that TGF-β production is IL-10 dependent.

In vivo studies show that plasma levels of both IL-10 and TGF-β were increased following Nx and correlated positively with their expression in the renal tissues. IL-10 content in both, plasma and renal tissues, decreased with the progression of CRG and returned to the normal levels during first few days postsurgery. It is interesting to note that reduction of IL-10 plasma levels occurs earlier than in the remaining kidney tissues. This phenomenon is most probably due to different clearance and consumption between plasma and tissues. It is conceivable therefore to expect different time curves when plasma levels and cortical expression are examined. Furthermore, plasma IL-10 derives from a variety of sources such as mononuclear and endothelial cells (30) in contrast to the cortical tissue.

In contrast to IL-10, TGF-β expression in the renal tissues and plasma was reduced within 24 h postsurgery, compared with the basal levels, and temporary increase from 72 h with a peak at 1 wk after nephrectomy. Thus the rise of IL-10 in plasma and kidney tissues appeared earlier than the increase in TGF-β. This suggests that upregulation of IL-10 may be important to the induction of TGF-β and subsequently to the development of compensatory tubular cell hypertrophy in vivo. In summary of these results, we suggest that mesangial cells initiate compensatory tubular cell hypertrophy via IL-10-induced TGF-β secretion.

In the next step, we studied the effect of AS101 on CRG. It has been found that AS101 inhibits IL-10 production by cultured mesangial cells isolated from either normal, sham, or remaining kidneys in a dose-dependent manner. In addition, it has been shown that AS101 also reduced the TGF-β contents in the mesangial supernatants in parallel with a reduction in IL-10. However, in the absence of IL-10 secretion, AS101 does not affect TGF-β levels. Indeed, in cultured mesangial cells isolated from normal kidneys, while the amounts of IL-10 were less than detectable levels, AS101 did not reduce the expression of TGF-β. These observations provided evidence that AS101 has no direct effect on TGF-β secretion and that AS101 inhibits TGF-β production by cultured mesangial cells through its inhibitory effect on IL-10. AS101 treatment to Nx rats results in a reduction in IL-10 content in plasma and in the remaining kidney at the initial phase of compensatory renal growth. This reduction was followed by a decrease in TGF-β content in the renal tissues and plasma. These events resulted in a 20–25% reduction in the weight of the remaining kidney and fractional kidney weight ratio and significantly decreased hypertrophy of proximal and distal tubular cells (protein/DNA ratio and morphometric studies). Although the link between the inhibition of IL-10/TGF-β pathway and the decrease of CRG by AS101 is not completely demonstrated in the absence of control with an anti-TGF-β, it has been shown by Ziyadeh (62) that treatment of diabetic animals with neutralizing anti-TGF-β antibodies prevents the development of mesangial matrix expansion and reduces tubular cell hypertrophy. In parallel, similar results were obtained in our study showing inhibitory effect of AS101 on TGF-β-induced compensatory tubular cell hypertrophy.

It is of interest that the mean glomerular surface area and the mean number of intraglomerular cells were unchanged in PBS-treated Nx rats, compared with sham-operated animals, and significantly reduced by AS101 treatment. The nature of this phenomenon is not clear and remains to be elucidated. We suggest that apoptosis of intraglomerular cells, which has a part in CRG (51), may contribute to this phenomenon.

The results stated here concerning AS101 support our assumption about the essential role of IL-10 in the development of compensatory renal growth. Unilateral nephrectomy increased IL-10 secretion by mesangial cells during the initial phase of CRG. These events led, in turn, to an increase in TGF-β production by mesangial cells and resulted in marked hypertrophy of the remaining kidney. Treatment of unilateral nephrectomized rats with AS101 through the inhibition of IL-10 and the subsequent reduction of TGF-β secretion by mesangial cells partially inhibit compensatory hypertrophy of the remaining kidney.

Studies have shown that IL-10 does not have a sole effect on TGF-β. TGF-β production might be induced by several factors. One of the most studied is ANG II, which stimulates the expression of TGF-β in proximal tubular cells, thus mediating tubular cell hypertrophy (26, 58, 59). In addition, there is circumstantial evidence that IGF-I and possibly growth hormone (directly or via IGF-I) are involved in both the transcriptional and posttranslational regulation of CRG (15). Moreover, there is a cross talk between IGF-I and TGF-β secretion (41, 42). This may explain that following significant inhibition of IL-10 activity by AS101, the renal tubular hypertrophy of the remaining kidneys was diminished only partially.

As known, compensatory renal growth is regulated by a variety of growth factors and cytokines, which initiate proliferative, hypertrophic, and apoptotic growth responses in the remaining kidneys (33, 40, 56). These growth factors may act in concert, and that, despite their apparent redundancy, they all must be present in sufficient concentrations to support maximal growth of the remaining kidney. For the reason of interdependence between cytokines, we suggest that manipulation of the levels of one of these factors may affect the entire compensatory growth response in the remaining kidney.

This study thus contributes to the understanding of processes leading to compensatory tubular cell hypertrophy and may suggest a potential therapeutic function for the immunomodulator AS101.

**GRANTS**

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