Gas6 Induces Mesangial Cell Proliferation via Latent Transcription Factor STAT3*

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Mesangial cell proliferation is essential for the pathogenesis and progression of glomerular disease. Previously, we showed that Gas6 plays a pivotal role in mesangial cell proliferation in vitro and in vivo. In the present study, we identified downstream targets of Gas6 signaling to examine the role in mesangial cell proliferation in vitro and in vivo. We found that Gas6 tyrosine phosphorylates STAT3 (signal transducers and activators of transcription) with concomitant translocation to the nucleus and induces STAT3-dependent transcriptional activation in cultured mesangial cells. Expressing dominant negative STAT3 inhibited Gas6-mediated transcriptional activation of STAT3 and abolished Gas6-induced mesangial cell proliferation. In a model of mesangial proliferative glomerulonephritis, STAT3 is phosphorylated in mesangial cells, and its phosphorylation peaks at day 8 after the injection of anti-Thy1.1 antibody. Inhibition of Gas6 by warfarin and the extracellular domain of Axl (a receptor for Gas6) abolishes mesangial cell proliferation by specific inhibition of the Gas6-mediated pathway in experimental glomerulonephritis (10). Moreover, administration of these agents abolishes the induction of PDGF-B in Thy1 GN. Thus, Gas6 seems to be not only a mitogen for mesangial cells, but also to play a crucial role in the progression of glomerular diseases by modulating the expression of other growth factors. It has been previously reported that constitutive activation of Eyk, a member of the Axl superfamily (11), results in cell transformation in vitro without significant stimulation of the Ras/ERK (extracellular signal-regulated kinase) pathway (11, 12), which is activated by many oncogenes in the process of cell transformation (13). The transforming activities of Eyk depend on phosphorylation of STAT3, a member of the STAT (signal transducers and activators of transcription) protein family. STAT proteins are latent transcription factors that become activated by phosphorylation of single tyrosine, which causes STAT proteins to dimerize. STAT dimerization is usually followed by translocation to the nucleus (14). Within the nucleus, STAT proteins recognize and bind to consensus DNA binding sites that represent enhancer sequences for a variety of genes, including the immediate early growth response genes. Activation of STAT proteins is often associated with differentiation and growth regulation. For example, STAT3 activation has been implicated in proliferation of cells (15), while STAT1 activation correlates with growth arrest of cells (16, 17).

To elucidate molecular mechanisms of Gas6-mediated mesangial cell proliferation, we sought to identify downstream signaling components of the Gas6/Axl pathway essential for its various activities. In this study, we report that STAT3 is a key signaling molecule in Gas6-mediated mesangial cell proliferation in vitro and in vivo. This study reveals the crucial role of STAT3 activation in the pathogenesis of experimental glomerulonephritis.

MATERIALS AND METHODS

Reagents and Antibodies—Anti-STAT3 and anti-phospho-STAT3 (Y705, 9131) polyclonal antibodies were from New England Biolabs (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and the enhanced chemiluminescent system were from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). FITC- and rhodamine-labeled goat anti-rabbit IgG were from Chemicon International.

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The abbreviations used are: PDGF, platelet-derived growth factor; STAT, signal transducers and activators of transcription; FITC, fluorescein isothiocyanate; SMA, smooth muscle actin; DME, Dulbecco's modified Eagle's medium; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; GN, glomerulonephritis.
FIG. 1. Gas6 stimulates tyrosine phosphorylation and nuclear translocation of STAT3. A, dose-dependent phosphorylation of STAT3 in response to Gas6. Mesangial cells were stimulated with the indicated concentrations of Gas6 for 10 min, and their lysates were subjected to immunoblotting with antibodies against STAT3 and phospho-STAT3. Representative data is shown from three independent experiments. pSTAT3, phospho-STAT3. The bands were analyzed with GS-800 Calibrated Densitometer and Quantity One. The fold increases in phosphorylation of STAT3 are shown below the blot (means ± S.D. for three independent experiments). B, time course of phosphorylation of STAT3 in response to Gas6. Mesangial cells were stimulated with 500 ng/ml of Gas6 for the indicated times, and their lysates were subjected to immunoblotting with antibodies against STAT3 and phospho-STAT3. Mesangial cells stimulated with PDGF-BB (250 ng/ml) for 10 min were used as a positive control for STAT3 phosphorylation. Representative data is shown from three independent experiments. The bands were analyzed with GS-800 Calibrated Densitometer and Quantity One. The fold increases in phosphorylation of STAT3 are shown below the blot (means ± S.D. for three independent experiments). C, inhibition of STAT3 phosphorylation by Axl-Fc. Mesangial cells were stimulated with vehicle (lane 1, 2) or Gas6 (500 ng/ml) (lane 3, 4) after preincubation (lane 2, 4) or without Axl-Fc (lane 1, 3). Lysates of these cells were subjected to immunoblotting with antibodies against STAT3 and phospho-STAT3. Representative data is shown from four independent experiments. D, Gas6-induced translocation of STAT3. Mouse mesangial cells were stimulated with Gas6 (500 ng/ml) for 0 min (a) and 10 min (b). Mesangial cells stimulated with PDGF-BB (250 ng/ml) were used as a positive control (c). After the stimulation, mesangial cells were stained with antibody against phospho-STAT3 followed by FITC labeled goat anti-rabbit IgG. Representative data is shown from four independent experiments.
were incubated for another 2 h. After serum starvation, mesangial cells were stimulated with Gas6 for 8 h. Whole cell lysates of mesangial cells (10 μg) were subjected to luciferase assays according to the manufacturer’s instructions.

Thymidine Incorporation with Dominant Negative STAT3—Mesangial cells (0.5 × 10^5) were transfected with 1 μg of an expression vector encoding dominant negative STAT3 (18) or a control vector, ReCMV using LipofectAMINE Plus. After 6 h, medium was changed to growth medium and incubated for 4 h. After incubation in starving medium for 24 h, mesangial cells were stimulated with Gas6 or PDGF for 22 h and pulse-labeled with [3H]thymidine for 2 h, and incorporated [3H]thymidine was measured as previously described (9). The transfection efficiency was about 60% by X-gal staining (not shown).

Induction of Experimental Mesangial Proliferative Glomerulonephritis (Thy1 GN)—Thy1 GN was induced by a single intravenous injection of mouse anti-Thy1.1 monoclonal antibody OKT-7 (1 mg/kg body weight) as described elsewhere (19, 20). Rats were sacrificed on days 0, 3, 5, 8, and 15.

Isolation of Glomeruli—Glomeruli were isolated from the renal cortex of rats using the differential sieving method (21, 22). The purity of the glomeruli was higher than 90%.

Histo logical Examinations—Kidney tissues were snap frozen in cold acetone in OCT compound and cryostat sections (4 μm) were stained using indirect immunofluorescence procedure with rabbit polyclonal antibodies against phospho-STAT3 (1:100 dilution). Detection of αSMA was done by direct immunofluorescence procedure with FITC-conjugated monoclonal antibody against αSMA (1A4). Specificity of the procedures was confirmed by substituting the primary antibody with equivalent concentrations of irrelevant murine monoclonal antibodies or rabbit preimmune IgG.

Immunofluorescent Double Staining—To determine the localization of phospho-STAT3, double immunostaining for αSMA and phospho-STAT3 was done. Both primary antibodies were incubated overnight at 4 °C followed by the incubation with rhodamine-conjugated anti-rabbit IgG. Specificity was confirmed by the negative results when replacing either one of the primary antibodies with irrelevant mouse monoclonal antibody or rabbit preimmune IgG. Specimens were observed with a Zeiss microscope equipped with proper filters.

Warfarin Treatment in Thy1 GN—Six rats were treated with warfarin in drinking water (0.5 mg/L) from 5 days before the initiation of Thy1 GN to the days of sacrifice as previously described (10). In this experiment, rats were sacrificed on day 8.

Treatment with Axl-Fc in Thy1 GN—Expression and purification of Axl-Fc was performed as previously described (9). Two hundred μg of recombinant Axl-Fc was intravenously administered to six rats once a day from 24 h after the injection of anti-Thy 1.1 antibodies to day 7 (10). In this experiment, rats were sacrificed on day 8.

Statistical Analysis—Statistical analyses were done by Student’s t test. Thymidine incorporation and luciferase activities were analyzed by two-way repeated analysis of variance followed by the Fisher’s post-hoc test. p values < 0.01 were considered significant. Data are expressed as means ± S.D. Analysis was performed by simple regression using the StatView program (Abacus Concepts Inc., Berkeley, CA).

RESULTS

Tyrosine Phosphorylation and Nuclear Translocation of STAT3 in Gas6-treated Mesangial Cells—To elucidate the role of STAT3 in Gas6-mediated mesangial cell proliferation, we examined whether Gas6 activates STAT3 by measuring its tyrosine phosphorylation and translocation to the nucleus in mouse mesangial cells. Cell lysates of mesangial cells treated with the indicated concentrations of Gas6 for 10 min were immunoblotted with anti-phospho-STAT3 and anti-STAT3 polyclonal antibodies. Gas6 increased phosphorylation of STAT3 in a dose-dependent manner and reached a peak at 500 ng/ml (Fig. 1A). In a time course experiment, tyrosine phosphorylation of STAT3 by 500 ng/ml of Gas6 increased by about 2.6-fold and peaked at 10 min (Fig. 1B). Next, we tested whether Gas6-induced phosphorylation of STAT3 was specifically mediated by Axl. We treated mesangial cells with Gas6 preincubated with 100 molar excess of the extracellular domain of Axl conjugated with the Fc portion of human IgG1 (Axl-Fc). Gas6-induced tyrosine phosphorylation of STAT3 was abolished by the preincubation of Gas6 with Axl-Fc (Fig. 1C), indicating that the phosphorylation of STAT3 is through the binding of Gas6 to the cell surface Axl. We also examined whether phosphorylated STAT3 (phospho-STAT3) is translocated to the nucleus in response to Gas6. In immunofluorescent analysis, phospho-STAT3 was almost undetectable in unstimulated mesangial cells (Fig. 1D) but became evident in the nuclear compartment 10 min after the stimulation with Gas6. PDGF-BB also induced nuclear translocation of phospho-STAT3.

Gas6 Stimulates STAT3-specific Transcription in Mesangial Cells—Next we examined whether Gas6 stimulates STAT3-specific transcription in mesangial cells by measuring the transcriptional activation of a reporter gene construct bearing four copies of the M67 site that is responsive to STAT3 activation (18). Transfection efficiencies were normalized by the renilla luciferase activities of pRLCMV. Mesangial cells transfected with control vector showed a 3.5-fold increase in luciferase activities when treated with Gas6, while mesangial cells transfected with dominant negative STAT3 showed a complete inhibition of STAT3-specific gene transcription induced by Gas6 (Fig. 2). Mesangial cells transfected with wild type STAT3 showed an increase in luciferase activities equivalent to that of control vector (data not shown). Viability of mesangial cells transfected with dominant negative STAT3 was examined by
measuring the levels of lactate dehydrogenase in the conditioned media and were not increased significantly compared with that of mesangial cells transfected with control vector or wild type STAT3 (data not shown). PDGF-BB increased STAT3-specific gene transcription by 2.5-fold in mesangial cells as previously described (23).

Role of STAT3 in Gas6-mediated Mesangial Cell Proliferation—To elucidate the role of STAT3 phosphorylation in Gas6-mediated cell proliferation, we examined whether Gas6-mediated cell proliferation depends on the phosphorylation of STAT3. Gas6 increased \[^{3}H\]thymidine incorporation by 3.2-folds in mesangial cells transfected with a control vector (Fig. 3). However, Gas6 did not increase \[^{3}H\]thymidine incorporation in the cells transfected with dominant negative STAT3, indicating that STAT3 is involved in mesangial cell proliferation induced by Gas6. In contrast, the cell proliferation by PDGF-BB was not affected by overexpressing dominant negative STAT3.

Role of STAT3 in Glomerular Cell Proliferation in Vivo—Since Gas6-mediated mesangial cell proliferation appears to depend on the phosphorylation of STAT3 in vitro, we examined the in vivo role of STAT3 phosphorylation in mesangial cell proliferation. We utilized an acute model of mesangial proliferative glomerulonephritis known as Thy1 glomerulonephritis (Thy1 GN) (10, 19, 20). In Thy1 GN, mesangial cell proliferation begins at day 2, peaks at day 8, and resolves by day 15 after the injection of anti-Thy1.1 antibody (10). First we examined the phosphorylation of STAT3 in glomerular proteins. The glomerular proteins collected on days 0, 3, 5, 8, and 15 were immunoblotted with anti-STAT3 and anti-phospho-STAT3 antibodies. Phosphorylation of STAT3 was almost undetectable on day 0 but became evident during the course of Thy1 glomerulonephritis, with a 5.3-fold increase on day 8 (Fig. 4A). Increased phosphorylation of STAT3 subsided on day 15. The expression level of STAT3 protein was not changed during the period. Localization of phospho-STAT3 was confirmed by immunostaining. Nuclei of glomerular cells on day 0 showed scarce staining of phospho-STAT3 (Fig. 4B). On day 3, nuclei began to be phosphorylated, and the phosphorylation level increased gradually in parallel with the activity of mesangial proliferation, peaked on day 8, and decreased on day 15. Simultaneously, double immunostaining for phospho-STAT3 and \(\alpha\)-SMA (a marker of activated mesangial cells) (24) was done to identify the cell types with nuclear staining of phospho-STAT3 in the glomerulus. Overlay of phospho-STAT3 and \(\alpha\)-SMA of kidney section on day 8 demonstrated that the majority of phospho-STAT3-positive nuclei are activated mesangial cells, indicating an active role of phospho-STAT3 in mesangial cell proliferation in vivo.
Gas6 and STAT3 in Mesangial Proliferation

In this study, we have demonstrated that STAT3 is a key signaling molecule in Gas6-mediated mesangial cell proliferation. We have shown that Gas6 stimulates phosphorylation of STAT3 on tyrosine and the phosphorylation of STAT3 is mediated through the cell surface receptor, Axl. We have also shown that Gas6 stimulates translocation of phospho-STAT3 to the nucleus and STAT3-specific transcription. Because tyrosine phosphorylation of STAT3 and nuclear translocation of phospho-STAT3 are shown to be necessary and sufficient for its activities (25), our data indicates that Gas6 stimulates the activation of STAT3 through Axl. Further we have shown that Gas6-mediated mesangial cell proliferation depends on activation of STAT3. We have extended these in vitro observations to an in vivo model of glomerulonephritis. In Thy1 GN, STAT3 is heavily phosphorylated in the nuclei of glomerular cells. This phosphorylation parallels the increase in mesangial cell proliferation and peaks at day 8 when the expression of Gas6 as well as mesangial cell proliferation peaks. Most of the cells with phospho-STAT3 were shown to be mesangial cells. Furthermore, interference of the Gas6-specific pathway by the administration of warfarin or Axl-Fc completely inhibited the phosphorylation of STAT3 in glomeruli as well as the mesangial cell proliferation. Thus, our study has identified STAT3 as a signaling molecule involved in the development of glomerulonephritis and implicated it as a novel target of the therapeutic strategy for kidney disease.

As we showed in a previous report, Gas6 plays a crucial role in the development of glomerulonephritis, and the expression of Gas6 in the mesangial region could be a marker showing the activities of mesangial proliferation and glomerulonephritis (10). In the present study, we have provided evidence that STAT3 is another marker of mesangial cell proliferation, because phosphorylation of STAT3 is increased in parallel with the activity of mesangial proliferation, and our immunofluorescent study revealed that the site of STAT3 phosphorylation is mainly in the glomerulus rather than in the tubulus, corresponding to the site of proliferation in Thy1 GN. Further, results of double immunostaining of phospho-STAT3 and αSMA indicate that the nuclei of mesangial cells are the sites of STAT3 phosphorylation. Thus, our data indicate that STAT3 is a pivotal mediator of mesangial cell proliferation. Therefore, it would be intriguing to examine whether phosphorylation of STAT3 can be detected in human glomerulonephritis, such as lupus nephritis and IgA glomerulonephritis, and whether phosphorylation is related to the activity and prognosis of glomerulonephritis.

In mouse mesangial cells, the transfection efficiency was about 60% with LipofectAMINE plus (data not shown). Despite the presence of about 40% of untransfected cells, Gas6-mediated mesangial cell proliferation is totally inhibited by the transfection of dominant negative STAT3. This result might be due to the effect of some unknown factors secreted from growth-arrested cells transfected with dominant negative STAT3 on the proliferation of untransfected cells. On the other hand, proliferative response to PDGF-BB was not affected by the transfection of dominant negative STAT3. Therefore, activation of STAT3 might not be necessary for the mitogenic activities of PDGF-BB in mesangial cells.

Activation of STAT3 by Eyk is considered to occur directly after the binding of STAT3 via its SH2 domain to the $^{933}$YVPQ

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**Fig. 5. Warfarin and Axl-Fc abrogates phosphorylation of STAT3.** A, glomerular lysates (60 μg/lane) from Thy1 rats of day 0, day 8 without treatment, day 8 with warfarin (W) or Axl-Fc treatment were subjected to immunoblotting with antibodies against STAT3 and phospho-STAT3. Representative data is shown from four individual experiments. pSTAT3, phospho-STAT3. B, immunostaining of phospho-STAT3 during the course of Thy1 glomerulonephritis. Kidney sections of Thy1 rats on day 0 (a), day 8 without treatment (b), day 8 with warfarin treatment (c), and day 8 with Axl-Fc treatment (d) were subjected to immunostaining with antibody against phospho-STAT3. Magnification, ×200.

**DISCUSSION**

In this study, we have demonstrated that STAT3 is a key signaling molecule in Gas6-mediated mesangial cell proliferation and peaks at day 8 when the expression of Gas6 as well as mesangial cell proliferation peaks. Most of the cells with phospho-STAT3 were shown to be mesangial cells. Furthermore, interference of the Gas6-specific pathway by the administration of warfarin or Axl-Fc completely inhibited the phosphorylation of STAT3 in glomeruli as well as the mesangial cell proliferation. Thus, our study has identified STAT3 as a signaling molecule involved in the development of glomerulonephritis and implicated it as a novel target of the therapeutic strategy for kidney disease.

It is intriguing to note that interference of the Gas6-mediated pathway in Thy1 GN completely inhibited the phosphorylation of STAT3 as well as mesangial cell proliferation, although other growth factors and cytokines (26–29) present in Thy1 GN can stimulate phosphorylation of STAT3 as well. Therefore, our findings indicate that Gas6-mediated STAT3 activation would be one of the initial events in the development of Thy1 GN and suggest that STAT3 is a critical signaling molecule of Gas6-mediated glomerular proliferation and might regulate the development of glomerular diseases by modulating the expression of other growth factors. It would be more important to study the role of Gas6 and STAT3 in a chronic model of glomerulonephritis.

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sequence present in v-Eyk and by subsequent tyrosine phosphorylation of STAT3 (11). Similar sequences (YXXQ, YLRQ) are also shown to be important for STAT3 activation in several cytokine receptors (30, 31). Axl does not contain YVSPQ, but YXXQ sequences (YYRQ, YLRQ) are present in its cytoplasmic domain (32). However, we could not demonstrate the direct binding of STAT3 to Axl (data not shown). One explanation may be because of the rapid and transient binding of STAT3 and Axl. As seen in Fig. 1C, phospho-STAT3 was rapidly translocated to the nucleus and could not be seen in the cytoplasm. The phenomenon was similar at 5 min after the addition of Gas6, and phospho-STAT3 could not be detected earlier than 5 min (data not shown).

In conclusion, our study establishes an important role for STAT3 in Gas6-mediated mesangial cell proliferation. These data led us to speculate that inhibition of STAT3 activation in vivo could limit the extent of mesangial cell proliferation. Recently, it was shown that inhibition of STAT3 activation by the administration of dominant negative STAT3 results in suppression of cell transformation and tumor progression in vivo (33, 34). A better understanding of the Gas6-STAT3 pathway could provide a basis for new and specific targets for therapeutic interventions in various kidney diseases associated with mesangial cell proliferation.

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