Mesangioproliferative Kidney Diseases and Platelet-Derived Growth Factor–Mediated AXL Phosphorylation

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Rationale & Objective: Immunoglobulin A nephropathy (IgAN) is a common glomerular disease, with mesangial cell proliferation as a major feature. There is no disease-specific treatment. Platelet-derived growth factor (PDGF) contributes to the pathogenesis of IgAN. To better understand its pathogenic mechanisms, we assessed PDGF-mediated AXL phosphorylation in human mesangial cells and kidney tissue biopsy specimens.

Study Design: Immunostaining using human kidney biopsy specimens and in vitro studies using primary human mesangial cells.

Setting & Participants: Phosphorylation of AXL was assessed in cultured mesangial cells and 10 kidney-biopsy specimens from 5 patients with IgAN, 3 with minimal change disease, 1 with membranous nephropathy, and 1 with mesangioproliferative glomerulonephritis (GN).

Predictor: Glomerular staining for phospho-AXL in kidney biopsy specimens of patients with mesangioproliferative diseases.

Outcomes: Phosphorylated AXL detected in biopsy tissues of patients with IgAN and mesangioproliferative GN and in cultured mesangial cells stimulated with PDGF.

Analytic Approach: t test, Mann-Whitney test, and analysis of variance were used to assess the significance of mesangial cell proliferative changes.

Results: Immunohistochemical staining revealed enhanced phosphorylation of glomerular AXL in IgAN and mesangioproliferative GN, but not in minimal change disease and membranous nephropathy. Confocal-microscopy immunofluorescence analysis indicated that mesangial cells rather than endothelial cells or podocytes expressed phospho-AXL. Kinomic profiling of primary mesangial cells treated with PDGF revealed activation of several protein-tyrosine kinases, including AXL. Immunoprecipitation experiments indicated an association of AXL and PDGF receptor proteins. An AXL-specific inhibitor (bemcentinib) partially blocked PDGF-induced cellular proliferation and reduced phosphorylation of AXL and PDGF receptor and the downstream signals (AKT1 and ERK1/2).

Limitations: Small number of kidney biopsy specimens to correlate the activation of AXL with disease severity.

Conclusions: PDGF-mediated signaling in mesangial cells involves transactivation of AXL. Finding appropriate inhibitors to block PDGF-mediated transactivation of AXL may provide new therapeutic options for mesangioproliferative kidney diseases such as IgAN.

Chronic kidney disease (CKD) represents a significant global health burden with a high economic cost to health systems because they afflict approximately one-seventh of adults older than 20 years.1 Glomerular diseases, a subset of CKD, are an important cause of morbidity and mortality. Mesangial–cell activation and extracellular–matrix expansion often occur in progressive glomerular diseases, such as immunoglobulin A nephropathy (IgAN). Activation of mesangial cells may involve multiple stimuli and signaling pathways with protein-tyrosine kinases; inhibition of some of these signaling pathways may reduce mesangial-cell proliferation.2

There is no disease-specific treatment for IgAN. Therapeutic measures to decrease proteinuria and maintain kidney functions are recommended by the KDIGO (Kidney Disease Improving Global Outcomes) guidelines.3 Supportive therapies to dampen the renin-angiotensin system are the current standard. With better understanding of the pathogenesis of IgAN, new therapeutic strategies have been proposed and are being tested, such as BAFF/TRAIL inhibitor atacicept,4 a distal ileum–targeted steroid budesonide,5 and SYK inhibitor fostamatinib.6

Activation of certain protein-tyrosine kinases, including platelet-derived growth factor (PDGF) receptor (PDGFR), epidermal growth factor receptor (EGFR), and vascular endothelial growth factor (VEGF), has been observed in cultured mesangial cells and animal models. Inhibitors of these protein-tyrosine kinases decrease the proliferation of mesangial cells and improve proteinuria and kidney function in animal models.2 However, the therapeutic effects of these inhibitors are not satisfactory and do not fulfill the requirement for clinical application.6 Further investigation of the signal transduction pathways, especially the network relationship of these tyrosine kinases related to mesangial cell proliferation, is required.

In this study, we assessed whether the protein-tyrosine kinase AXL is transactivated by PDGF, as reported in some cancers.7,8 AXL is a receptor protein-tyrosine kinase originally identified in cancer cells.9 It belongs to a TAM (TYRO3, AXL, and MERTK) family of receptor protein-

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tyrosine kinases and growth arrest–specific 6 is one of its ligands. In pathologic conditions, AXL is overexpressed and is associated with cancer cell proliferation, survival, evasion from apoptosis, and resistance to targeted anti-cancer therapies. 

PDGF is a cellular mitogen and chemoattractant with many essential physiologic and pathophysiologic functions. Four genes encode 4 PDGF subunits: A, B, C, and D. The active forms of PDGF are dimers (PDGF-AA, -AB, -BB, -CC, and -DD), for which biological effects are mediated by cell-surface PDGFRs, PDGFR-α and/or -β. Upon ligand binding, PDGFR undergoes dimerization to form homo- or heterodimers (PDGFR-αα, -αβ, and -ββ). This process activates intracellular tyrosine-kinase domains, resulting in PDGFR phosphorylation and downstream recruiting/signaling events.

PDGF plays a role in the pathogenesis of IgAN, and PDGF mesangial overexpression has been reported in several other kidney diseases. Mesangial cells are regulated by PDGFs in both physiologic and pathologic processes. Human mesangial cells express PDGF-A and -B and their receptors. Notably, the degree of expression of PDGF-AB and -BB parallels the severity of proliferative glomerular changes in IgAN. Furthermore, PDGFR-β is overexpressed in IgAN, correlating with mesangial cell proliferation. Thus, mounting evidence indicates that PDGFR-β is involved in the pathogenesis of mesangio-proliferative glomerulonephritis (GN), such as IgAN. However, the mechanisms of activation of mesangial cells by PDGF-AB are not fully understood.

In this study, we examined activation of AXL in kidney biopsy specimens from patients with mesangio-proliferative glomerular diseases. We next assessed PDGF-induced signaling in primary human mesangial cells through their main PDGFR, PDGFR-β. By using kinomic profiling, immunoprecipitation, immunodetection, and signaling inhibitor, we found that AXL, a receptor protein-tyrosine kinase, was transactivated by PDGF-AB. These findings have implications for potential therapeutic approaches focused on interference with PDGF-PDGFR signaling pathways in IgAN and other types of mesangio-proliferative GN.

**METHODS**

**Materials**

All chemicals, unless specified otherwise, were purchased from Sigma Aldrich; cell culture supplies were purchased from Gibco.

**Immunohistochemistry**

Normal human mesangial cells were purchased from Lonza. Mesangial cells from patients with IgAN were isolated from fresh remnant kidney biopsy specimens. Cells were cultured in a chamber slide (Nunc Lab-TekII Chamber Slide System, Thermo Fisher Scientific) to reach 20% to 30% confluence. Cells were then fixed with 4% paraformaldehyde for 10 minutes, washed with phosphate-buffered saline (PBS), and permeabilized with 0.5% Triton X-100 for 1 hour. After a wash with PBS containing 0.5% Tween 20 (PBST), goat anti-human AXL polyclonal antibody (1:200, R&D Systems) was added and incubated overnight at 4 °C followed by donkey anti-goat IgG antibody conjugated with Alexa Fluor 568 (1:100; Invitrogen; Thermo Fisher Scientific). Nuclei were stained with Hoechst 33342 (10 mg/mL in PBS) for 5 minutes. After a wash with PBST, chambers were removed, cover slips were mounted, and stained tissues were examined with a Zeiss Axioplan 2 microscope equipped with a Zeiss Axiocam digital camera.

Formalin-fixed and paraffin-embedded remnant kidney biopsy specimens from patients with minimal change disease (MCD), IgAN, membranous nephropathy, and mesangio-proliferative GN admitted to Shanghai Hospital were used. The Shanghai Changhai Hospital Ethics Committee approved this study (#CHEC2020-002). Because the specimens were remnant kidney biopsy tissues no longer needed for diagnosis, were not collected for this study, and no personal identifier was revealed, the study was exempt from obtaining written informed consent from the patients whose biopsy specimens were used. Tissue sections were deparaffinized, hydrated, and heated for 10 minutes at 120 °C before being blocked with 10% fetal bovine serum (FBS) for 10 minutes. Tissue sections were then incubated overnight at 4 °C with rabbit polyclonal anti–human Phospho-AXL (Y779) antibody (R&D Systems) followed by detection with anti-rabbit HRP-DAB Cell & Tissue Staining Kit (R&D Systems). Tissues were counterstained with hematoxylin.
**Staining of Frozen-Tissue Sections From Kidney Biopsy Specimens**

Remnant frozen kidney biopsy specimens from patients with IgAN or MCD were stained for phospho-AXL (P-AXL; R&D Systems), Ulex europaeus lectin (Vector Laboratories), and synaptopodin (a kind gift from Peter Mundel) for localization of P-AXL in glomeruli. Sections were incubated with 0.3% Triton X-100 for 5 minutes and then blocked for 1 hour (blocking solution, PBS with 2% bovine serum albumin, 2% FBS, and 0.2% fish gelatin) before being incubated with P-AXL antibody, 1:100 for 2 hours, followed by Alexa-488–conjugated secondary antibody. Synaptopodin (1 hour) or Ulex europaeus lectin (1:300; 30 minutes) was used for costaining. Images were taken using a Zeiss LSM 800 confocal microscope.

**Cell Culture**

Primary human mesangial cells were purchased from Lonza. Cells (passage 3) were expanded in RPMI 1640 medium with 20% FBS in a 5% carbon dioxide–humidified incubator. For signal transduction studies and immunoprecipitation, mesangial cells were serum-starved for 48 hours in RPMI 1640 medium with 0.5% FBS. When reaching 80% to 85% confluence, cultured cells were used in cellular proliferation assays, kinomic studies, immunoprecipitation, and immunofluorescence staining.

**Mesangial Cell Proliferation Assay**

Primary human mesangial cells were plated in 96-well cell culture black-well plates (BD Biosciences) at 5×10^3/well in RPMI 1640 medium with 20% FBS. After 24 hours, cells were quiescent in RPMI 1640 medium with 0.5% FBS and incubated for 22 to 24 hours. Next, the cells were stimulated with 10 ng/mL of human PDGF-AB (R&D Systems) in fresh RPMI 1640 medium with 0.5% FBS in the presence or absence of AXL inhibitor R428 (APExBIO). BrdU cell proliferation assay was performed after incubation with PDGF-AB with or without inhibitors for 22 hours according to manufacturer’s instructions (Roche Diagnostics).

**Immunoprecipitation and Western Blotting**

To assess the association of PDGFR and AXL, serum-starved normal human mesangial cells were stimulated with 10 ng/mL of PDGF-AB for 15 minutes. Cells were washed with ice-cold PBS and lyed on ice with M-PER lysis buffer (Pierce Biotechnology, Inc) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific). The resultant cell lysates were centrifuged at 20,000g for 10 minutes at 4 °C and the supernatants were used for further analyses. Protein concentration was measured using Bio-Rad protein assay kit (Bio-Rad Laboratories). After preclearing with Protein A-Sepharose, the lysates (100 μg of protein) were incubated with 2 μg of either rabbit monoclonal antibody specific for AXL (C89E7; Cell Signaling Technology) or mouse monoclonal antibody against PDGFR-β (2B3; Cell Signaling Technology) overnight at 4 °C with rotation. Twenty-five microliters of washed Protein G agarose (50% protein G-agarose slurry per 100 μg of protein was added to each sample, incubated at 4 °C for 4 hours with rotation. Protein G agarose was then pelleted by centrifugation and washed 3 times with washing buffer (20 mmol/L of Tris/HCL, pH 7.4, 150 mmol/L of sodium chloride, 10% glycerol, and 1% Triton X-100 with protease and phosphatase inhibitors). Protein G–bound material was extracted with 30 μL of 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 minutes. After centrifugation, the supernatant was analyzed by SDS-PAGE under reducing conditions. The separated proteins were then electroblotted to polyvinylidene difluoride (PVDF) membrane. After blocking with blocking buffer, membranes were probed with biotin-labeled polyclonal goat anti-human AXL or rabbit anti-human PDGFR antibodies, followed by corresponding horseradish peroxidase–conjugated secondary antibodies (Southern Biotech). Membranes were developed with enhanced chemiluminescence reagent (ECL Western Blotting Substrate; Pierce Biotechnology Inc) and signal detected using x-ray film.

**Signal Transduction Experiments**

Quiescent mesangial cells were stimulated with PDGF-AB in the presence or absence of AXL inhibitor R428 for 15 minutes. Cells were then washed and lysed with M-PER lysis buffer supplemented with protease and phosphatase inhibitors and the lysates were processed as described. Immunoblotting used antibodies specific for PDGFR-β (R&D Systems), phospho-PDGFR-β (Cell Signaling Technology), ERK1/2 (Cell Signaling Technology), phospo-ERK1/2 (R&D Systems), AKT1 (Santa Cruz Technology), phospho-AKT1 (Santa Cruz Technology), AXL, and phospho-AXL (R&D Systems), and the blots were developed as described.

**Kinomic Profiling**

Lysates (15 μg of protein) prepared from mesangial cells stimulated with 10 ng/mL of PDGF-AB for 15 minutes were subjected to protein-tyrosine kinome analysis using high-throughput phospho-peptide microarrays, as described earlier, using the UAB Kinome Core. The 15-minute stimulation was chosen, based on a time course experiment with immunoblotting using a phospho-tyrosine antibody (PY20). Lysates from cells without PDGF-AB were used as a negative control. All analyses were performed in triplicate on the PamStation 12 platform (PamGene). This platform uses a high-throughput peptide microarray system analyzing 144 individual tyrosine phosphorylatable peptides imprinted and immobilized in a 3-dimensional format to assess kinomic activity in cell lysates. Fluorescein isothiocyanate–conjugated anti-phospho-tyrosine antibodies were used for visualization.
Capture of peptide phosphorylation signals was monitored through a computer-controlled charge-coupled device. Kinomic profiling was analyzed using Evolve microarray software (PamGene) for initial sample and array processing as well as image capture and BioNavigator software (PamGene) for raw data transformation into kinetic (initial velocity) and steady-state (postwash) values across multiple exposure times. Significantly altered peptide lists (based on unpaired t test) from cell lysates and differences between samples from control cells and PDGF-AB treated cells were generated.

Statistical Analysis

Statistical significance between 2 data sets was assessed using t test, 2 tailed, or the Mann-Whitney test. Analysis of variance was used to determine differences in characteristics among multiple groups. Data were expressed as mean ± standard deviation or median values. P < 0.05 was considered significant. All statistical analyses were performed with SPSS, version 21.0, software (IBM Inc).

RESULTS

AXL Phosphorylation in Glomeruli of Patients with IgAN and Mesangio proliferative GN

There was enhanced staining of phospho-AXL in tissue sections from patients with IgAN and mesangio proliferative GN (MsPGN). Immunohistochemical staining of kidney biopsy specimens from patients with different glomerular diseases revealed that phospho-AXL is overexpressed in glomeruli of patients with IgAN (examples of glomeruli from 3 patients shown) and MsPGN but not in biopsy specimens from patients with minimal change disease (MCD) and membranous nephropathy (MN). Size bars (black lines) mark 50 μm.

Figure 1. Phospho-AXL expression in glomeruli of kidney-biopsy specimens from patients with immunoglobulin A nephropathy (IgAN) and mesangio proliferative glomerulonephritis (MsPGN). Immunohistochemical staining of kidney biopsy specimens from patients with different glomerular diseases revealed that phospho-AXL is overexpressed in glomeruli of patients with IgAN (examples of glomeruli from 3 patients shown) and MsPGN but not in biopsy specimens from patients with minimal change disease (MCD) and membranous nephropathy (MN). Size bars (black lines) mark 50 μm.

During and after lysates were pumped through the array, Capture of peptide phosphorylation signals was monitored through a computer-controlled charge-coupled device. Kinomic profiling was analyzed using Evolve microarray software (PamGene) for initial sample and array processing as well as image capture and BioNavigator software (PamGene) for raw data transformation into kinetic (initial velocity) and steady-state (postwash) values across multiple exposure times. Significantly altered peptide lists (based on unpaired t test) from cell lysates and differences between samples from control cells and PDGF-AB treated cells were generated.

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RESULTS

AXL Phosphorylation in Glomeruli of Patients with IgAN and Mesangio proliferative GN

There was enhanced staining of phospho-AXL in tissue sections from patients with IgAN and mesangio proliferative GN (Fig 1). The staining was localized to glomeruli and proximal tubules. In tissues from patients with MCD and membranous nephropathy, no glomerular staining was apparent. Thus, AXL was activated in glomeruli of 5 patients with mesangio proliferative glomerular diseases, IgAN and mesangio proliferative GN.

To expand the immunohistochemistry data, we next used immunofluorescence with frozen tissue sections to assess which cells in the glomeruli produce phospho-AXL. Immunofluorescence staining of the tissues of patients with IgAN revealed a mesangial pattern of phospho-AXL staining (Fig 2). Phospho-AXL staining did not colocalize with that for a marker for podocytes (synaptopodin) or endothelial cells (lectin from Ulex europeus), indicating that phospho-AXL was not expressed to any great extent in those cells. Phospho-AXL had much stronger expression in
kidney biopsy specimens from patients with IgAN than in those from patients with MCD (Fig 2).

**PDGF Activation of AXL and ABL in Mesangial Cells**

Because glomerular expression of PDGF-AB correlates with the severity of mesangioproliferative changes in kidney biopsy specimens of patients with IgAN, we used protein-tyrosine kinome profiling to better understand how PDGF-AB activates mesangial cells. The results revealed that AXL and ABL were the top-ranked protein-tyrosine kinases activated by PDGF-AB in mesangial cells (Fig 3A). In addition to AXL and ABL, other protein-tyrosine kinases, such as LTK, HCK, and TYK2, were activated by PDGF-AB (Fig 3A). SDS-PAGE with Western blot analysis with antibodies against TYRO3, AXL, and MERTK confirmed that AXL was the major TAM-family protein-tyrosine kinase expressed in normal human mesangial cells (Fig 3B), as well as in mesangial cells derived from a kidney biopsy specimen of a patient with IgAN (Fig 3C).

**PDGF-AB and Phosphorylation of AXL**

To confirm and extend our findings from protein-tyrosine-kinase profiling, we next tested whether PDGF can induce AXL phosphorylation. Immunoblotting with antibodies

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**Figure 2.** Overexpressed phospho-AXL in human glomeruli in immunoglobulin A nephropathy (IgAN) biopsy samples in relation to markers for podocytes and endothelial cells. Immunofluorescence staining of sections of remnant frozen kidney biopsy specimens from patients with IgAN revealed a mesangial pattern of phospho-AXL staining. Phospho-AXL (green) did not colocalize with markers for podocytes (A) synaptopodin (red) and (B) endothelial cells, *Ulex europeus* lectin (red), indicating that phospho-AXL is not expressed to any great extent in those cells. Phospho-AXL had stronger expression in kidney biopsy specimens from patients with IgAN than in biopsy specimens from patients with minimal change disease (MCD). Images were taken with a ×40 objective.
against AXL and phospho-AXL (Y779) showed that PDGF-AB induced phosphorylation of AXL at tyrosine 779 (Fig 4).

Association of AXL and PDGFR-β in Mesangial Cells

Because PDGF-AB is not a ligand for AXL, we hypothesized that AXL may be transactivated by PDGF-AB. For example, AXL may form heterodimers with PDGFR-β, as reported for AXL and EGF receptor.7,25 To test this hypothesis, we used immunoprecipitation with antibodies specific for AXL and PDGFR-β, respectively. PDGFR-β was pulled down with AXL by an antibody specific for AXL (Fig 5). Conversely, AXL was pulled down with PDGFR-β by an antibody specific for PDGFR-β (Fig 5). These findings suggest that fractions of each of these 2 proteins, AXL and PDGFR-β, are associated (Fig 5). As expected, PDGF-AB induced phosphorylation of PDGFR-β, and immunoprecipitation with antibody against PDGFR-β showed that it also pulled down AXL phosphorylated at Y779. Conversely, an antibody specific for AXL pulled down a phosphorylated form of PDGFR-β (Y751; Fig 5). Control samples from normal human mesangial cells not stimulated with PDGF-AB did not

Figure 3. AXL activation by PDGF. (A) Kinomic studies revealed that multiple protein-tyrosine kinases were activated in primary normal human mesangial cells (NHMC) during a 15-minute stimulation with platelet-derived growth factor (PDGF)-AB (10 ng/mL). Protein-tyrosine kinases identified by kinomic profiling and analyzed with protein-tyrosine kinase UpKin, version 8.0 (BioNavigator), software are listed on the y-axis with the normalized kinase statistic scores shown on the x-axis. The bar color of protein-tyrosine kinases indicates activities induced by PDGF-AB, showing highest activities in red. This kinomic profiling showed that the kinase activity of AXL, a member of TAM family, was top ranked. (B) Western blot analysis of cell lysates using antibodies against the 3 members of the TAM family, TYRO3, AXL, MERTK, revealed that AXL was the major protein of TAM family expressed in NHMC. Molecular weights of the standard proteins in kDa are shown on the side. (C) Immunofluorescence staining for AXL in primary human mesangial cells. AXL was expressed in NHMC as well as in mesangial cells isolated from kidney-biopsy specimens from patients with immunoglobulin A nephropathy (IgAN). Three samples were used in each group; representative images are shown. Negative control is without primary antibody; only nuclei are stained (blue).
show phosphorylation of AXL (Y779) in any pull-down sample regardless of the antibody used for immunoprecipitation. These results indicate association of AXL and PDGFR-β proteins and suggest that phosphorylation of AXL by PDGF-AB may occur by transactivation with PDGFR-β.

**AXL Inhibitor R428 Inhibition of Cellular Proliferation of Mesangial Cells Induced by PDGF-AB**

PDGF-AB (10 ng/mL) added to normal human mesangial cells increased cellular proliferation by more than 3-fold (Fig 6). Bemcentinib (R428) is a specific AXL inhibitor; it exhibited dose-dependent partial inhibition of the PDGF-induced cellular proliferation (P = 0.02 for 0 vs 0.3 μmol/L of R428; Fig 6). R428 did not alter the baseline cellular proliferation of control normal human mesangial cells (ie, without PDGF; Fig 6).

**DISCUSSION**

Expression of PDGF and PDGFR is tightly controlled in adulthood such that exaggerated PDGF-PDGFR signaling, except in wound repair and healing, is considered abnormal. Increased PDGF-PDGFR expression and signaling is involved in many diseases involving cellular proliferation, including kidney diseases with mesangio-proliferative lesions and some types of cancer and inflammation.

We confirmed that stimulation of mesangial cells with PDGF-AB increased cellular proliferation. Treatment with...
PDGF-AB induced phosphorylation of PDGFR-β and the downstream signaling, leading to cellular proliferation. In addition, kinomic profiling of mesangial cells stimulated with PDGF-AB showed activation of several additional protein-tyrosine kinases, of which AXL was the top-ranked activated protein-tyrosine kinase. Although other protein-tyrosine kinases were also activated by PDGF-AB to a lesser degree, we sought to define how AXL is activated by PDGF in mesangial cells.

AXL is a receptor protein-tyrosine kinase originally identified in cancer cells.9 It belongs to a TAM (TYRO3, AXL, and MERTK) family of receptor protein-tyrosine kinases, and growth arrest–specific 6 (Gas6) is one of its ligands.10 AXL is linked to epithelial-mesenchymal transition in kidney injury and promotes cell survival.19 In pathologic conditions such as in many types of cancers, AXL is overexpressed and is associated with cancer cell proliferation, survival, evasion from apoptosis, and resistance to targeted anticancer therapies.11 AXL is also overexpressed by mesangial cells in experimental proliferative GN.30

AXL was expressed by cultured primary human mesangial cells. Stimulation of these cells with PDGF led to phosphorylation of AXL and PDGFR-β. Immunoprecipitation experiments indicated a physical association of AXL with PDGFR-β, including their phosphorylated variants. We speculate that AXL in mesangial cells forms heterodimers with PDGFR-β and thus is transactivated by PDGF-AB. A similar mechanism has been reported in cancer cells for AXL and EGF receptor and, to a lesser degree, several other receptors, including PDGFR.7,25 In mesangial cells stimulated with PDGF, another protein-tyrosine kinase, ABL, was also activated (Fig 3A). ABL, a nonreceptor tyrosine kinase, is an intracellular regulator of cellular proliferation, differentiation, migration, and survival/death. ABL activation may be induced by signals from the PDGF/PDGFR pathway and from transactivated AXL.31 However, signal exchange between ABL and AXL is bidirectional. Signals from activated ABL may also induce phosphorylation of AXL.32 Our studies with R428, a specific AXL inhibitor,26,27 indicated that activation of AXL may exert synergistic effects to promote cellular proliferation in response to PDGF stimulation. Blocking this pathway may inhibit the proliferation of mesangial cells induced by PDGF and partially block PDGF-induced phosphorylation of PDGFR-β by inhibition signals from AXL to ABL.

Protein-tyrosine kinase crosstalk/transactivation at the receptor level33-36 may include formation of a heterodimer that can split downstream signals to 2 different branches/pathways. Examples of such branched pathways include

**Figure 6.** AXL inhibitor R428 partial inhibition of cellular proliferation of mesangial cells induced by platelet-derived growth factor (PDGF)-AB. AXL inhibitor R428 reduced in a dose-dependent manner the cellular proliferation of normal human mesangial cells (NHMC) induced by PDGF-AB (10 ng/mL; black bars). R428 did not alter the cellular proliferation of control NHMC (open bars). Results are shown as individual data points and mean and standard deviation values calculated from 3 independent experiments with duplicates. Blue dots are results from groups treated with R428. Black dots are results from groups treated with R428 and PDGF-AB. Statistical differences were determined by 1-way analysis of variance test ($P=0.002$ for 0 vs 0.3 μmol/L of R428).

**Figure 7.** AXL inhibitor R428 inhibition of platelet-derived growth factor (PDGF)-induced phosphorylation of AXL and PDGF receptor (PDGFR-β) and the downstream signaling to AKT1 and ERK1/2. Our previous experiment showed that R428, an AXL inhibitor, partially inhibited PDGF-induced cellular proliferation. Here, we assessed the capacity of R428 to block cellular signaling induced by PDGF. Normal human mesangial cells were preincubated with AXL inhibitor R428 (0.3 μmol/L) for 1.5 hour and then stimulated with PDGF-AB (10 ng/mL) for 15 minutes. The solvent for R428, dimethyl sulfoxide (DMSO), was used as an additional control. Cell lysates were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. R428 inhibited PDGF-induced phosphorylation of AXL as well as PDGFR-β. Downstream signaling to AKT1 and ERK1/2 was also inhibited by R428. White lines separate the noncontiguous parts of blots. Example of 1 of 2 independent experiments with similar results is shown. Molecular weights of the proteins are shown on the side (130, 180, 70, and 44/42kDa).
nonreceptor protein-tyrosine kinases, such as Src\(^{32,37-39}\) and ABL.\(^{32,38}\) Thus, AXL has the capacity to channel signals by transactivation/crosstalk with multiple protein-tyrosine kinases. These interactions may create complicated network signals that promote dysregulated cellular proliferation or drug resistance through multiple pathways in some cancers or inflammatory diseases.\(^{40,41}\)

We found enhanced phospho-AXL expression in kidney-biopsy tissue specimens from patients with IgAN and mesangioproliferative GN but not in specimens from patients with two nonproliferative glomerular diseases, MCD and membranous nephropathy. Thus, glomerular disorders other than IgAN and mesangioproliferative GN could be similarly affected. Immunofluorescence studies showed that phospho-AXL existed in mesangial cells but not in podocytes or endothelial cells. These findings are consistent with other reports of AXL expression in the kidney.\(^{39,42}\) In lupus nephritis, the extracellular domain of overexpressed AXL may be cleaved and released into circulation.\(^{43}\) In these patients, serum AXL level correlates with disease severity and kidney outcome. Conversely, serum AXL levels decrease during treatment of lupus nephritis and the decrement correlated with clinical responses and kidney histology-based responses.\(^{44}\) AXL is overexpressed in kidney–allograft–infiltrating cells in acute kidney rejection.\(^{45}\) Based on the findings in our study, the AXL pathway may be involved in IgAN pathogenesis through amplification of PDGF-driven signals. Thus, dampening the AXL pathway may lessen the kidney injury in IgAN and other proliferative glomerular diseases. For example, in a mouse model of anti–glomerular basement membrane nephritis, administration of R428 improved kidney function and decreased proliferation of glomerular mesangial cells.\(^{46}\) By inhibiting key regulators of fibrosis, R428 also decreases kidney fibrosis induced by unilateral ureteral obstruction in mice.\(^{47}\) Other AXL pathway regulators/inhibitors inhibit inflammation and fibrosis in animal models of acute kidney injury and glomerulonephritis induced with immunotoxin.\(^{48,49}\)

In summary, we found that PDGF-mediated activation of mesangial cells involves transactivation of AXL. Because we detected phosphorylated AXL in kidney biopsy specimens from patients with IgAN, AXL-mediated signaling may play a role in the kidney injury in this and other mesangioproliferative kidney diseases. Thus, future approaches for the treatment of IgAN and other mesangioproliferative kidney diseases will benefit from a better understanding of PDGF-PDGFR signaling and AXL transactivation.

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Methods and Cohort

Immunostaining – phospho-AXL

10 kidney biopsy specimens

5 IgA nephropathy (IgAN)
3 Minimal change disease
1 Mesangioproliferative GN (MPGN)
1 Membranous nephropathy (MN)

Outcomes

↑Phospho-AXL

IgAN

No phospho-AXL

MPGN

Immunostaining for phospho-AXL

Mesangial cells

Confocal microscopy

Immunofluorescence

Endothelial cells

Podocytes

Conclusion: PDGF-mediated signaling in mesangial cells involves transactivation of AXL. Finding appropriate inhibitors to block this PDGF-mediated transactivation of AXL may provide new therapeutic options for mesangioproliferative kidney diseases, such as IgAN.

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