Kidney Medicine

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 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.

Visual Abstract included

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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.[2]

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-

PLAIN-LANGUAGE SUMMARY

Immunoglobulin A (IgA) nephropathy is a common kidney disease worldwide. There is no disease-specific treatment and 20% to 40% of patients progress to kidney failure. Platelet-derived growth factor (PDGF) and its receptor, PDGFR, play a role in IgA nephropathy by activating kidney mesangial cells. This study assessed the mechanisms of PDGF-mediated activation of mesangial cells. PDGF activated multiple protein-tyrosine kinases relevant to cellular proliferation. One of the kinases, AXL, activated through crosstalk with PDGFR, provides signals for cellular proliferation. An AXL inhibitor reduced AXL cross-activations and cellular proliferation. Activated AXL was detected in kidney biopsy specimens from patients with IgA nephropathy, indicating that AXL may be involved in mesangioproliferative kidney diseases such as IgA nephropathy.

tyrosine kinases and growth arrest–specific 6 is one of its ligands.[10] In pathologic conditions, AXL is overexpressed and is associated with cancer cell proliferation, survival, evasion from apoptosis, and resistance to targeted anticancer therapies.[11]

PDGF is a cellular mitogen and chemoattractant with many essential physiologic and pathophysiologic functions.[12] 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.[13] Upon ligand binding, PDGFR undergoes dimerization to form homo- or heterodimers (PDGFR-αα, -αβ, and -ββ).[13] This process activates intracellular tyrosine-kinase domains, resulting in PDGFR phosphorylation and downstream recruiting/signaling events.[13]

PDGF plays a role in the pathogenesis of IgAN,[14] and PDGF mesangial overexpression has been reported in several other kidney diseases.[13,15-17] Mesangial cells are regulated by PDGFs in both physiologic and pathologic processes.[18,19] 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.[14] Furthermore, PDGFR-β is overexpressed in IgAN, correlating with mesangial cell proliferation.[20] Thus, mounting evidence indicates that PDGFR-β is involved in the pathogenesis of mesangioproliferative glomerulonephritis (GN), such as IgAN.[17,21] 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 mesangioproliferative 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 mesangioproliferative 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.[22] 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 mesangioproliferative GN admitted to Changhai 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 europeus* 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 europeus* 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.[23] 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 lysed 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 proteinase 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), phospho-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.[24] 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

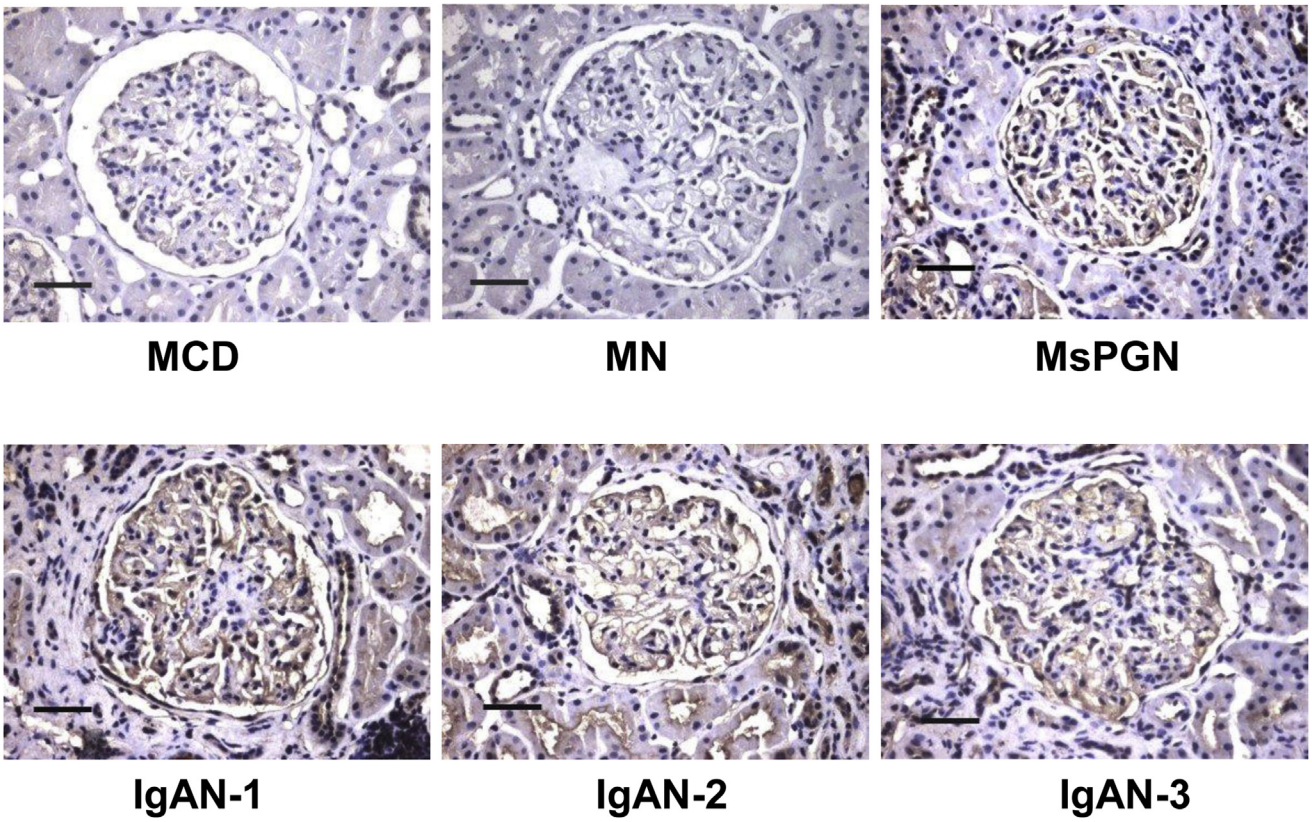

Figure 1. Phospho-AXL expression in glomeruli of kidney-biopsy specimens from patients with immunoglobulin A nephropathy (IgAN) and mesangioproliferative glomerulonephritis (MsPGN). Immunohistochemical staining of kidney biopsy specimens from patients with different glomerular diseases revealed that 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.

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 Mesangioproliferative GN

There was enhanced staining of phospho-AXL in tissue sections from patients with IgAN and mesangioproliferative 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 mesangioproliferative glomerular diseases, IgAN and mesangioproliferative 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

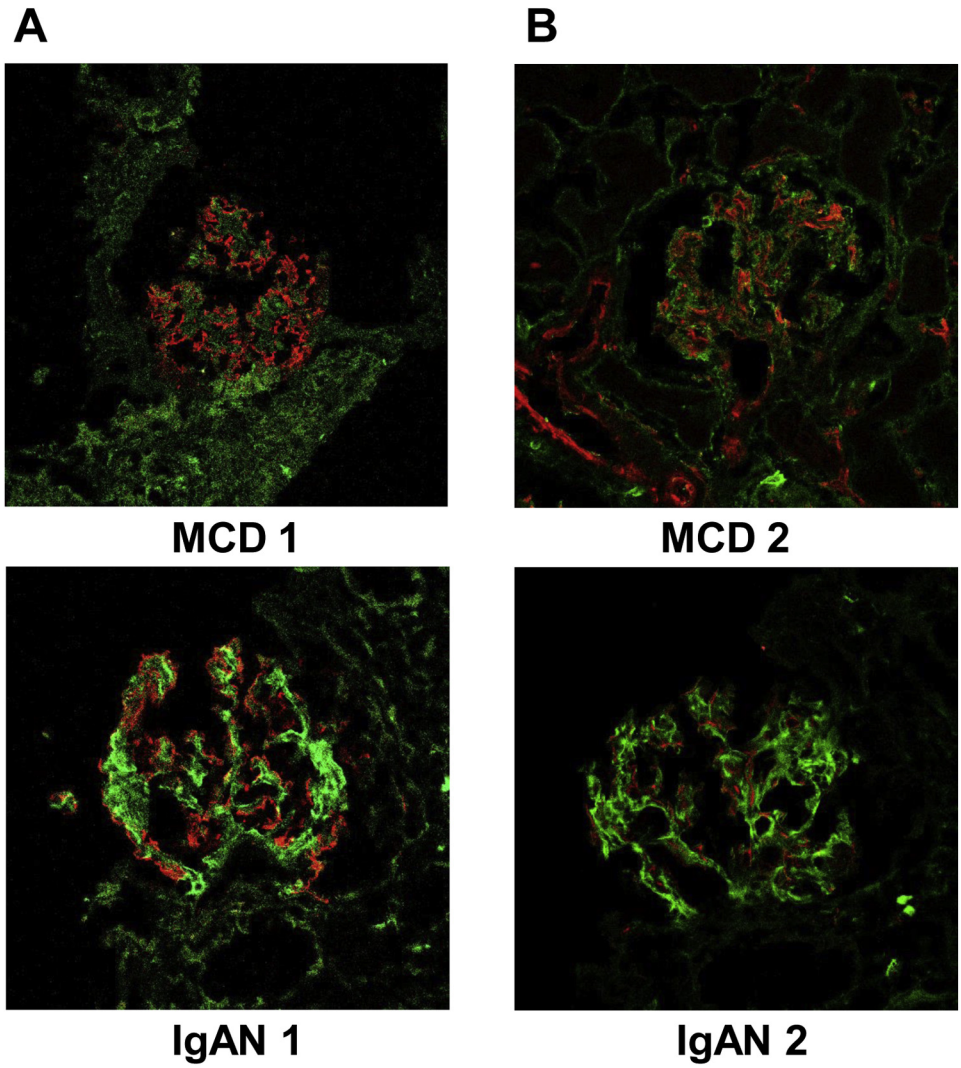

Figure 2. Overexpressed phospho-AXL in human glomeruli in immunoglobulin A nephropathy (IgAN) biopsy samples in relation to markers for podocytes and endothelial cells. Immunofluorescence staining of sections of remnant frozen kidney biopsy specimens from patients with IgAN revealed a mesangial pattern 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.

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,[14] 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

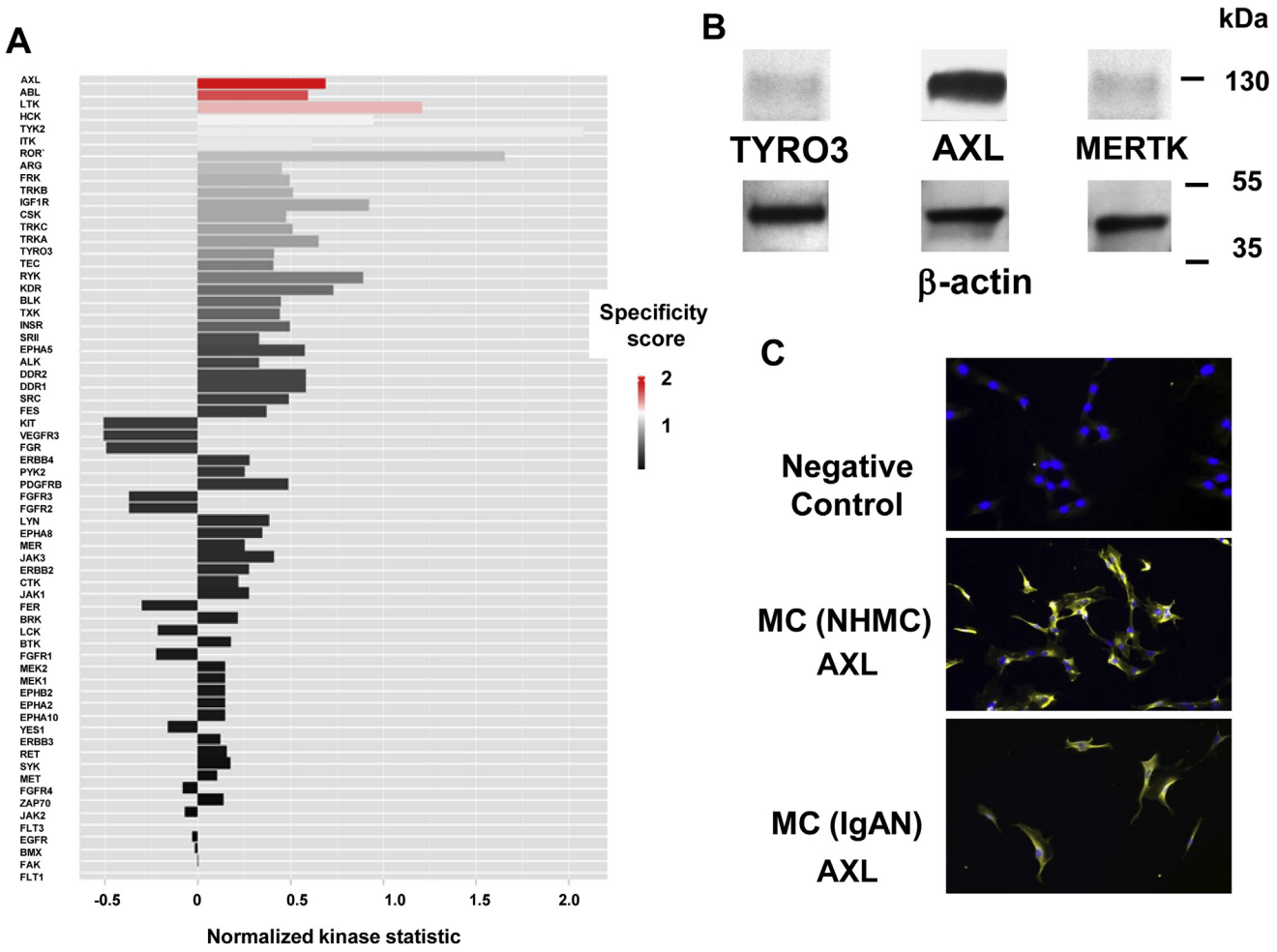

Figure 3. AXL activation by PDGF. (A) Kinomic studies revealed that multiple protein-tyrosine kinases were activated in primary normal human mesangial cells (NHMC) during a 15-minute stimulation with platelet-derived growth factor (PDGF)-AB (10 ng/mL). Protein-tyrosine kinases identified by kinomic profiling 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).

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

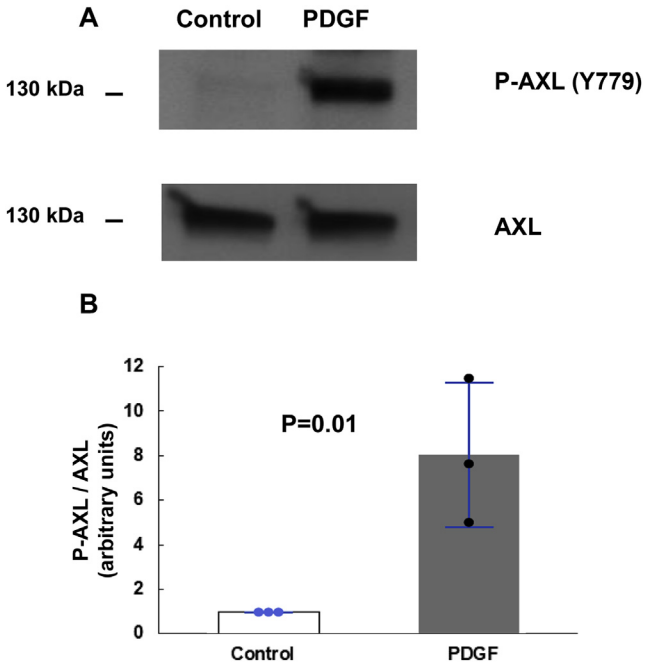

Figure 4. Platelet-derived growth factor (PDGF) activation of AXL in primary human mesangial cells. Cell lysates prepared from normal human mesangial cells (NHMC) after a 15-minute stimulation with 10 ng/mL of PDGF-AB or mock-stimulation (Control) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions followed by Western blotting with AXL- or phospho-AXL (P-AXL; Y779)-specific antibodies. (A) Typical example of a Western blot. Molecular weight of the standard protein (130 kDa) is shown on the side. These data were then evaluated by densitometry. (B) Individual data points and mean and standard deviation values calculated from 3 independent experiments ($P = 0.01$). Black dots are the results of groups treated with PDGF-AB. Blue dots are the results of groups without treatment. AXL was phosphorylated after a 15-minute stimulation of NHMC with PDGF-AB.

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[26,27]; 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).

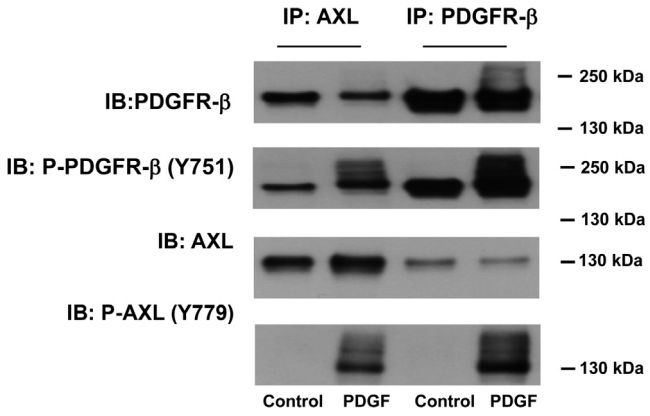

Figure 5. Platelet-derived growth factor (PDGF) activation of PDGF receptor (PDGFR)-β and cross-activation of AXL. Immunoprecipitation (IP) of cell lysates from normal human mesangial cells stimulated with PDGF-AB for 15 minutes. Antibodies specific for AXL or PDGFR-β were used for IP and the resultant material was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The blots were probed with antibodies specific for PDGFR-β, P-PDGFR-β, AXL, and P-AXL. PDGFR-β and AXL were co-precipitated by antibodies specific for AXL or PDGFR-β, suggesting that a fraction of each protein was associated with the other protein. Furthermore, PDGF-AB activated PDGFR-β and cross-activated AXL, as evidenced by the reactivity with antibodies specific for phosphorylated forms of AXL (Y779) and PDGFR-β (Y751). Abbreviations: AXL, immunoprecipitations with AXL-specific antibody; IB, immunoblotting; IP: PDGFR-β, immunoprecipitations with PDGFR-β–specific antibody. Example of 1 of 2 independent experiments with similar results is shown. Molecular weights of the standard proteins (130 and 250 kDa) are shown on the side.

AXL Inhibitor R428 Inhibition of PDGF-Induced Phosphorylation of AXL and PDGFR-β and Downstream Signaling to AKT1 and ERK1/2

Because AXL inhibitor R428 partially inhibited PDGF-induced cellular proliferation, we next tested the capacity of R428 to block cellular signaling induced by PDGF. R428 at a 0.3-μmol/L concentration reduced phosphorylation of AXL, PDGFR-β, AKT1, and ERK1/2 induced by PDGF (Fig 7). This finding confirmed the capacity of the AXL inhibitor to exert multiple effects on PDGF-AB–induced signaling cascade in primary human mesangial cells.

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 mesangioproliferative lesions and some types of cancer and inflammation.[28]

We confirmed that stimulation of mesangial cells with PDGF-AB increased cellular proliferation. Treatment with

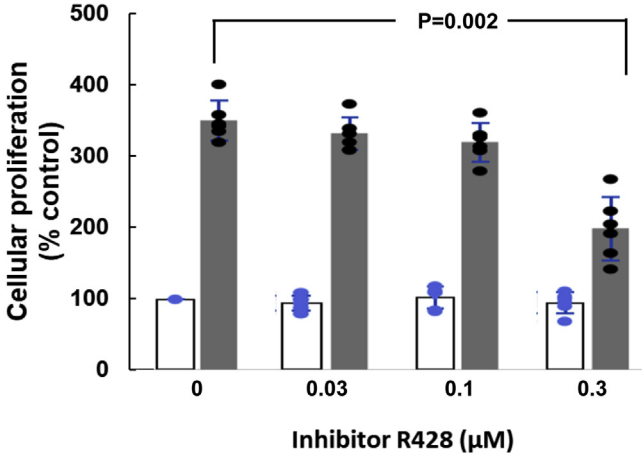

Figure 6. AXL inhibitor R428 partial inhibition of cellular proliferation of mesangial cells induced by platelet-derived growth factor (PDGF)-AB. AXL inhibitor R428 reduced in a dose-dependent manner the cellular proliferation of normal human mesangial cells (NHMC) induced by PDGF-AB (10 ng/mL; black 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).

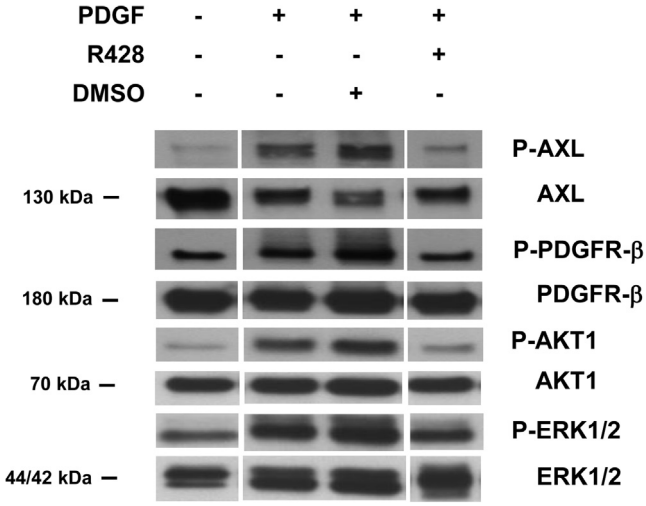

Figure 7. AXL inhibitor R428 inhibition of platelet-derived growth factor (PDGF)-induced phosphorylation of AXL and PDGF receptor (PDGFR)-β and the downstream signaling to AKT1 and ERK1/2. Our previous experiment showed that R428, an AXL inhibitor, partially inhibited PDGF-induced cellular proliferation. 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).

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.[29] 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 level[33-36] may include formation of a heterodimer that can split downstream signals to 2 different branches/pathways. Examples of such branched pathways include

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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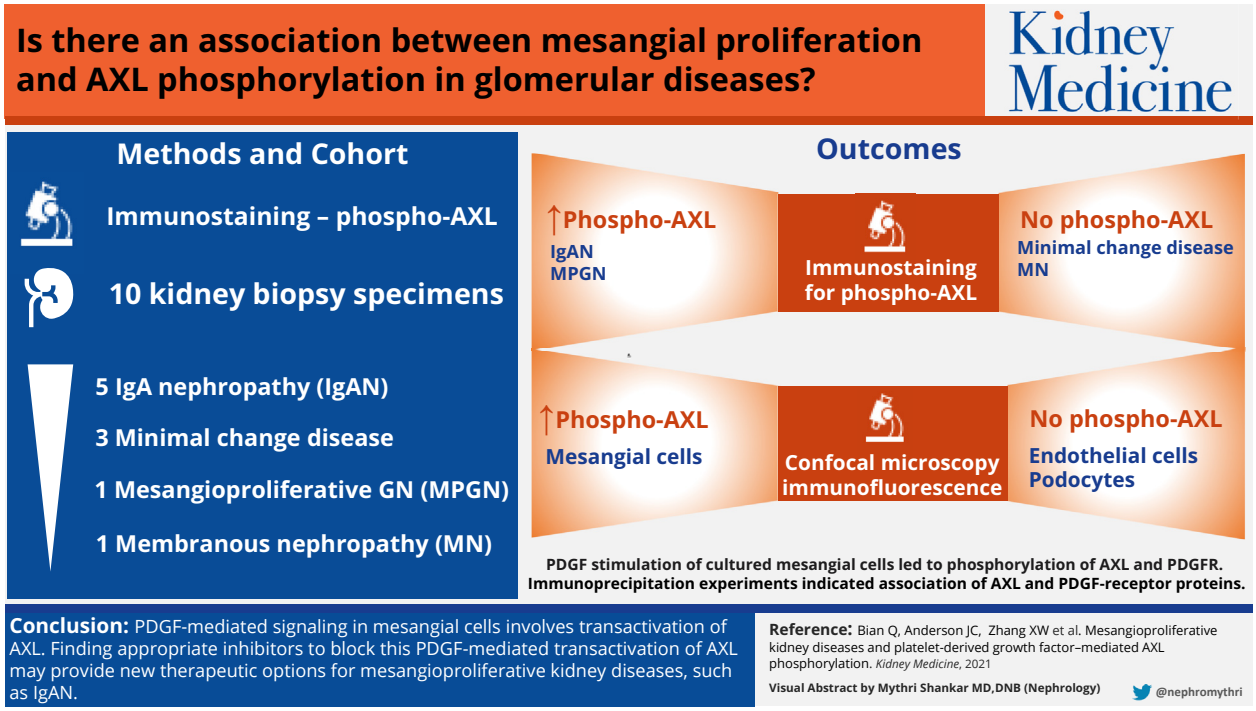
REFERENCES

1. Global Burden of Disease Study 2013 Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. 2015;386(9995):743-800.
2. Ma TK, McAdoo SP, Tam FW. Targeting the tyrosine kinase signalling pathways for treatment of immune-mediated glomerulonephritis: from bench to bedside and beyond. *Nephrol Dial Transplant*. 2017;32(suppl 1):i129-i138.
3. Floege J, Barbour SJ, Cattran DC, et al. Management and treatment of glomerular diseases (part 1): conclusions from a Kidney Disease: Improving Global Outcomes (KDIGO) Controversies Conference. *Kidney Int*. 2019;95(2):268-280.
4. Samy E, Wax S, Huard B, Hess H, Schneider P. Targeting BAFF and APRIL in systemic lupus erythematosus and other antibody-associated diseases. *Int Rev Immunol*. 2017;36(1):3-19.
5. Fellstrom B, Barratt J, Floge J, Jardine A. Targeted-release budesonide therapy for IgA nephropathy - authors' reply. *Lancet*. 2017;390(10113):2625-2626.
6. Kobayashi T, Furukawa Y, Kikuchi J, et al. Transactivation of RON receptor tyrosine kinase by interaction with PDGF receptor beta during steady-state growth of human mesangial cells. *Kidney Int*. 2009;75(11):1173-1183.

7. Meyer AS, Miller MA, Gertler FB, Lauffenburger DA. The receptor AXL diversifies EGFR signaling and limits the response to EGFR-targeted inhibitors in triple-negative breast cancer cells. *Sci Signal*. 2013;6(287):ra66.
8. Scaltriti M, Elkabets M, Baselga J. Molecular pathways: AXL, a membrane receptor mediator of resistance to therapy. *Clin Cancer Res*. 2016;22(6):1313-1317.
9. O'Bryan JP, Frye RA, Cogswell PC, et al. AXL, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. *Mol Cell Biol*. 1991;11(10):5016-5031.
10. Graham DK, DeRyckere D, Davies KD, Earp HS. The TAM family: phosphatidylserine sensing receptor tyrosine kinases gone awry in cancer. *Nat Rev Cancer*. 2014;14(12):769-785.
11. Brown M, Black JR, Sharma R, Stebbing J, Pinato DJ. Gene of the month: Axl. *J Clin Pathol*. 2016;69(5):391-397.
12. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev*. 1999;79(4):1283-1316.
13. Boor P, Ostendorf T, Floege J. PDGF and the progression of renal disease. *Nephrol Dial Transplant*. 2014;29(suppl 1):i45-i54.
14. Niemir ZI, Stein H, Noronha IL, et al. PDGF and TGF-beta contribute to the natural course of human IgA glomerulonephritis. *Kidney Int*. 1995;48(5):1530-1541.
15. Uehara G, Suzuki D, Toyoda M, Umezono T, Sakai H. Glomerular expression of platelet-derived growth factor (PDGF)-A, -B chain and PDGF receptor-alpha, -beta in human diabetic nephropathy. *Clin Exp Nephrol*. 2004;8(1):36-42.
16. Floege J, Eitner F, Alpers CE. A new look at platelet-derived growth factor in renal disease. *J Am Soc Nephrol*. 2008;19(1):12-23.
17. Nakagawa T, Inoue H, Sasahara M. Platelet-derived growth factor and renal disease. *Curr Opin Nephrol Hypertens*. 2012;21(1):80-85.
18. Doi T, Vlassara H, Kirstein M, Yamada Y, Striker GE, Striker LJ. Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor. *Proc Natl Acad Sci U S A*. 1992;89(7):2873-2877.
19. Johnson RJ, Floege J, Couser WG, Alpers CE. Role of platelet-derived growth factor in glomerular disease. *J Am Soc Nephrol*. 1993;4(2):119-128.
20. Ranieri E, Gesualdo L, Grandaliano G, Maiorano E, Schena FP. The role of alpha-smooth muscle actin and platelet-derived growth factor-beta receptor in the progression of renal damage in human IgA nephropathy. *J Nephrol*. 2001;14(4):253-262.
21. Terada Y, Yamada T, Nakashima O, et al. Expression of PDGF and PDGF receptor mRNA in glomeruli in IgA nephropathy. *J Am Soc Nephrol*. 1997;8(5):817-819.
22. Ebefors K, Liu P, Lassen E, et al. Mesangial cells from patients with IgA nephropathy have increased susceptibility to galactose-deficient IgA1. *BMC Nephrol*. 2016;17:40.
23. Novak J, Raskova Kafkova L, Suzuki H, et al. IgA1 immune complexes from pediatric patients with IgA nephropathy activate cultured human mesangial cells. *Nephrol Dial Transplant*. 2011;26(11):3451-3457.
24. Yamada K, Huang ZQ, Raska M, et al. Inhibition of STAT3 signaling reduces IgA1 autoantigen production in IgA nephropathy. *Kidney Int Rep*. 2017;2(6):1194-1207.
25. Vouri M, Croucher DR, Kennedy SP, An Q, Pilkington GJ, Hafizi S. Axl-EGFR receptor tyrosine kinase hetero-interaction provides EGFR with access to pro-invasive signalling in cancer cells. *Oncogenesis*. 2016;5(10):e266.
26. Holland SJ, Pan A, Franci C, et al. R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Res*. 2010;70(4):1544-1554.
27. Ghosh AK, Secreto C, Boysen J, et al. The novel receptor tyrosine kinase Axl is constitutively active in B-cell chronic lymphocytic leukemia and acts as a docking site of nonreceptor kinases: implications for therapy. *Blood*. 2011;117(6):1928-1937.
28. Chen PH, Chen X, He X. Platelet-derived growth factors and their receptors: structural and functional perspectives. *Biochim Biophys Acta*. 2013;1834(10):2176-2186.
29. Antony J, Huang RY. AXL-driven EMT state as a targetable conduit in cancer. *Cancer Res*. 2017;77(14):3725-3732.
30. Lu Y, Ye Y, Yang Q, Shi S. Single-cell RNA-sequence analysis of mouse glomerular mesangial cells uncovers mesangial cell essential genes. *Kidney Int*. 2017;92(2):504-513.
31. Wang JY. The capable ABL: what is its biological function? *Mol Cell Biol*. 2014;34(7):1188-1197.
32. Hoj JP, Mayro B, Pendergast AM. A TAZ-AXL-ABL2 feed-forward signaling axis promotes lung adenocarcinoma brain metastasis. *Cell Rep*. 2019;29(11):3421-3434 e8.
33. Yeh CY, Shin SM, Yeh HH, et al. Transcriptional activation of the Axl and PDGFR-alpha by c-Met through a ras- and Src-independent mechanism in human bladder cancer. *BMC Cancer*. 2011;11:139.
34. Gujral TS, Karp RL, Finski A, et al. Profiling phospho-signaling networks in breast cancer using reverse-phase protein arrays. *Oncogene*. 2013;32(29):3470-3476.
35. Salian-Mehta S, Xu M, Wierman ME. AXL and MET crosstalk to promote gonadotropin releasing hormone (GnRH) neuronal cell migration and survival. *Mol Cell Endocrinol*. 2013;374(1-2):92-100.
36. Revach OY, Sandler O, Samuels Y, Geiger B. Cross-talk between receptor tyrosine kinases AXL and ERBB3 regulates invadopodia formation in melanoma cells. *Cancer Res*. 2019;79(10):2634-2648.
37. Guo A, Villen J, Kornhauser J, et al. Signaling networks assembled by oncogenic EGFR and c-Met. *Proc Natl Acad Sci U S A*. 2008;105(2):692-697.
38. Sirvent A, Benistant C, Roche S. Cytoplasmic signalling by the c-Abl tyrosine kinase in normal and cancer cells. *Biol Cell*. 2008;100(11):617-631.
39. Volinsky N, Kholodenko BN. Complexity of receptor tyrosine kinase signal processing. *Cold Spring Harb Perspect Biol*. 2013;5(8):a009043.
40. Shi X, Wang B. Caught in the "Akt": cross-talk between EphA2 and EGFR through the Akt-PIKfyve axis maintains cellular sensitivity to EGF. *Sci Signal*. 2018;11(541):eaau1207.
41. Stallaert W, Bruggemann Y, Sabet O, Baak L, Gattiglio M, Bastiaens PIH. Contact inhibitory Eph signaling suppresses EGF-promoted cell migration by decoupling EGFR activity from vesicular recycling. *Sci Signal*. 2018;11(541):eaat0114.
42. Li S, Guo Q, Zhu H, Li Z, Su Y, Dong B. Increased Mer and Axl receptor tyrosine kinase expression on glomeruli in lupus nephritis. *Clin Rheumatol*. 2017;36(5):1063-1070.
43. Orme JJ, Du Y, Vanarsa K, et al. Heightened cleavage of Axl receptor tyrosine kinase by ADAM metalloproteases may contribute to disease pathogenesis in SLE. *Clin Immunol*. 2016;169:58-68.
44. Parodis I, Ding H, Zickert A, et al. Serum Axl predicts histology-based response to induction therapy and long-term renal outcome in lupus nephritis. *PLoS One*. 2019;14(2):e0212068.
45. Dangi A, Natesh NR, Husain I, et al. Single cell transcriptomics of mouse kidney transplants reveals a myeloid cell pathway for transplant rejection. *JCI Insight*. 2020;5(20):e141321.

46. Zhen Y, Lee IJ, Finkelman FD, Shao WH. Targeted inhibition of Axl receptor tyrosine kinase ameliorates anti-GBM-induced lupus-like nephritis. *J Autoimmun*. 2018;93:37-44.
47. Landolt L, Furriol J, Babickova J, et al. AXL targeting reduces fibrosis development in experimental unilateral ureteral obstruction. *Physiol Rep*. 2019;7(10):e14091.
48. Chen DQ, Feng YL, Chen L, et al. Poricoic acid A enhances melatonin inhibition of AKI-to-CKD transition by regulating Gas6/AxlNFkappaB/Nrf2 axis. *Free Radic Biol Med*. 2019;134:484-497.
49. Kurata A, Tachibana Y, Takahashi T, Horiba N. Novel AXL-specific inhibitor ameliorates kidney dysfunction through the inhibition of epithelial-to-mesenchymal transition of renal tubular cells. *PLoS One*. 2020;15(4):e0232055.


Is there an association between mesangial proliferation and AXL phosphorylation in glomerular diseases?
Kidney Medicine
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
MPGN
Immunostaining for phospho-AXL
No phospho-AXL
Minimal change disease
MN
↑Phospho-AXL
Mesangial cells
Confocal microscopy immunofluorescence
No phospho-AXL
Endothelial cells
Podocytes
PDGF stimulation of cultured mesangial cells led to phosphorylation of AXL and PDGFR. Immunoprecipitation experiments indicated association of AXL and PDGF-receptor proteins.
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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