S100A10 Is a Critical Mediator of GAS6/AXL-Induced Angiogenesis in Renal Cell Carcinoma

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Abstract

Angiogenesis is a hallmark of cancer that promotes tumor progression and metastasis. However, antiangiogenic agents have limited efficacy in cancer therapy due to the development of resistance. In clear cell renal cell carcinoma (ccRCC), AXL expression is associated with antiangiogenic resistance and poor survival. Here, we establish a role for GAS6/AXL signaling in promoting the angiogenic potential of ccRCC cells through the regulation of the plasminogen receptor S100A10. Genetic and therapeutic inhibition of AXL signaling in ccRCC tumor xenografts reduced tumor vessel density and growth under the renal capsule. GAS6/AXL signaling activated the expression of S100A10 through SRC to promote plasmin production, endothelial cell invasion, and angiogenesis. Importantly, treatment with the small molecule AXL inhibitor cabozantinib or an ultra-high affinity soluble AXL Fc fusion decoy receptor (sAXL) reduced the growth of a pazopanib-resistant ccRCC patient-derived xenograft. Moreover, the combination of sAXL synergized with pazopanib and axitinib to reduce ccRCC patient-derived xenograft growth and vessel density. These findings highlight a role for AXL/S100A10 signaling in mediating the angiogenic potential of ccRCC cells and support the combination of AXL inhibitors with antiangiogenic agents for advanced ccRCC.

Significance: These findings show that angiogenesis in renal cell carcinoma (RCC) is regulated through AXL/S100A10 signaling and support the combination of AXL inhibitors with antiangiogenic agents for the treatment of RCC.

Introduction

Renal cell carcinoma is a common malignancy with 403,262 new cases and 175,098 deaths worldwide in 2018 (1). Clear cell renal cell carcinoma (ccRCC) is the most common form of kidney cancer and is associated with loss of the von Hippel Lindau (VHL) tumor suppressor. VHL loss results in the constitutive activation of the hypoxia inducible transcription factors (HIF-1 and HIF-2) and their targets, including the proangiogenic factors VEGF and PDGF (2). As a result, RCC tumors are highly vascularized and initially respond to antiangiogenic therapies, including tyrosine kinase inhibitors (TKI; ref. 3). Although antiangiogenic therapy initially respond to antiangiogenic therapies, including tyrosine kinase inhibitors (TKI; ref. 3). Although antiangiogenic therapy eventually become resistant and progress (4, 5). Thus, antiangiogenic drug resistance is a major challenge in the clinical management of renal cell carcinoma. Multiple mechanisms of acquired resistance to antiangiogenic agents have been proposed in ccRCC, including the activation of compensatory angiogenesis mechanisms and increased tumor invasion (6, 7). The identification of druggable TKI resistance mechanisms in ccRCC are needed to improve the overall survival rate of patients with advanced kidney cancer.

The receptor tyrosine kinase, AXL, has emerged as an important therapeutic target in cancer that is associated with both metastatic and drug resistant phenotypes of advanced tumors. Moreover, multiple AXL inhibitors have advanced to clinical studies, highlighting the translational potential of targeting AXL signaling for cancer therapy (8–10). In ccRCC, AXL is a direct target of VHL/HIF signaling and its expression correlates with the lethal phenotype (11–13). Moreover, AXL expression is increased in sunitinib treated ccRCC patient tumors (14). The majority of AXL activation in ccRCC cells occurs in a ligand-dependent manner mediated by GAS6 (11). In cancer, GAS6/AXL signaling can be activated in an autocrine or paracrine manner with tumor cells as well as cells within the tumor microenvironment, including macrophages and endothelial cells producing biologically relevant sources of GAS6 (15). Analysis of GAS6 expression and AXL activation in a panel of ccRCC cells revealed that both autocrine and paracrine mechanisms are responsible for activation of AXL in these cells (11). Although GAS6/AXL signaling is known to promote the invasive and metastatic potential of tumor cells, the role of GAS6/AXL signaling in regulating the angiogenic potential of tumor cells is not known (11–13).

In this report, we establish a role for GAS6/AXL signaling in promoting the angiogenic potential of ccRCC cells through the regulation of S100A10. Genetic inhibition of AXL in ccRCC cells reduced tumor vessel density and growth under the renal
capsule. RNA sequencing (RNA-seq) analysis of AXL wild-type and AXL-deficient cells revealed that AXL promotes the expression of the plasminogen receptor S100A10. We demonstrate that the proangiogenic factor S100A10 is increased in ccRCC cells through AXL/SRC signaling. Moreover, S100A10 in ccRCC cells is sufficient to promote AXL-mediated plasmin production, endothelial invasion, and angiogenesis. In patients with ccRCC, S100A10 expression correlates with AXL expression. Finally, therapeutic blockade of GAS6/AXL signaling reduced ccRCC and patient-derived xenograft (PDX) tumor vessel density and growth in the kidney. Our findings identify GAS6/AXL signaling as an important pathway driving ccRCC angiogenesis and have important therapeutic implications for the treatment of advanced renal clear cell carcinoma.

Materials and Methods

Cell lines and culture conditions

786-O and M62 cells were maintained in DMEM supplemented with 10% FBS. HUVEC (ATCC CRL-1730) cells were purchased from ATCC and cultured in endothelial culture medium (CC-3156, LONZA) supplemented with Growth Medium 2 Supplement (C-39211, PromoCell). The M62 clear cell carcinoma cell line was a generous gift from Jose Karam and colleagues (MD Anderson Cancer Center, Houston, TX; ref. 16). For hypoxia treatments, cells were plated at the desired density 12 hours before placement in a hypoxia chamber (Innovo2-400; Ruskin Technologis) maintained at 2% oxygen for 0 to 72 hours, depending on the experiment. The M62 cell line expresses endogenous GAS6, whereas the 786-O cell line does not express endogenous GAS6 (11). Therefore, for all in vitro experiments, cells were pretreated with 200 ng/mL of recombinant human GAS6 (carrier free, 885-GS-050; R&D Systems) with >90% purity and <1.0 EU/1 µg of endotoxin for 24 hours before plating into the individual in vitro assays described below.

All cell lines were authenticated from the original source and were used within 6 months of receipt. In addition, cells were tested upon receipt for viability, cell morphology, and the presence of Mycoplasma and viruses (Charles River Laboratories).

Conditioned media

Forty-eight hours after transfection of ccRCC cells, the cells were treated with 200 ng/mL of GAS6 in serum-free medium for 24 hours. The medium was collected and centrifuged at 12,000 × g for 10 minutes. The supernatant was used for the HUVEC Matrigel invasion and in vivo Matrigel plug assays.

Correlation of transcript levels of AXL, ANXA2, and S100A10 in ccRCC patient samples

Gene expression profile of a cohort of 76 patients with ccRCC were retrieved from Gene Expression Omnibus (GSE36985). Expression levels of AXL, ANXA2, and S100A10 were extracted and log2 transformed in Excel. Log2 transformed expression levels were then centered and scatter plot was generated in Excel. Pearson correlation coefficient was calculated in Prism 6.

The Cancer Genome Atlas data analysis

The cBio Cancer Genomic Portal was used to analyze AXL, S100A10, and ANXA2 expression and survival in the Kidney Renal Clear Cell Carcinoma dataset (17). Consensus cluster analysis was performed using ConsensusClusterPlus (18).

shRNA and siRNA

Oligos for AXL shRNA were synthesized as previously described (11). For more details, please see the Supplementary Methods section.

IHC

The primary antibodies used for IHC staining are AXL (1:100, AF154, R&D Systems), S100A10 (1:200, 4E7E10, Santa Cruz Biotechnology), CD31 (1:200, ab28364, Abcam), Kpa70 (1:200, AB18560, Abcam), CAIX (1:500, NB-100-417, Novus), CD117 (1:200, CME296, Biocare Medical), CD10 (1:200, CM129AK, Biocare Medical), and CK7 (1:200, OV-TL 12/30, Dako Agilent). For more details, please see Supplementary Methods section.

Real-time PCR

RNA was isolated using TRIzol (Invitrogen) and subsequently treated with DNase 1 (Thermo). First-strand cDNA synthesis was performed with SuperScript II Reverse Transcriptase and random primers (Applied Biosystems) according to the manufacturer’s instructions. Quantitative real-time PCR was carried out using Power SYBR Green Master Mix (Bio-Rad), detection and data analysis were executed with the 7900HT Fast Real-Time PCR System (Applied Biosystems) by computing the results relative to a standard curve made with cDNA pooled from all samples, normalized to 18S. For more details and primer sequences, please see Supplementary Methods section.

Recombinant protein production

The soluble AXL (sAXL) Fc fusion proteins were generated as previously described (19). The sAXL (AVB500) was kindly provided by ARAVIVE Inc and is currently being tested in Phase Ib clinical trials.

Plasminogen activity assay

The kinetics of cell-mediated plasminogen activation was determined by measuring amidolytic activity of the plasmin generated from plasminogen (20). Cells (1 × 10⁴) were seeded on 96-well culture plates and stimulated with 200 ng/mL of recombinant human GAS6 in serum-free media overnight. Cells were washed with PBS and incubated with plasmin substrate (V0882, Sigma) at a final concentration of 100 µmol/L in phenol red-free and serum free DMEM. The reaction was initiated by the addition of 0.5 µmol/L [Glu]-plasminogen (528180, EMD Millipore) to the cells. The rate of plasmin production was measured at 405 nm with PerkinElmer HTS 7000 Bioassay reader at indicated time point.

Matrigel invasion

HUVEC cells were cultured in endothelial culture medium (CC-3156, LONZA) supplemented with Growth Medium 2 Supplement (C-39211, PromoCell). Matrigel invasion chambers with 8.0-µm pore polyester membrane were primed with 500 µL of endothelial basal culture medium. The reaction was initiated by the addition of 500 µL of [Glu]-plasminogen (528180, EMD Millipore) to the wells. Invasion was detected by crystal violet staining after 24 hours of incubation. For more details, please see Supplementary Methods section.
Protein isolation and Western blot analysis

Protein lysates were harvested in 9 mol/L Urea, 0.075 mol/L Tris buffer (pH 7.6). Protein lysates were quantified using the BCA Protein Assay Kit (Pierce), and 50 to 100 μg of protein was subjected to reducing SDS/PAGE using standard methods, then transferred onto 0.2 μm supported nitrocellulose membranes (Bio-Rad Laboratories). Western blots were probed with antibodies against AXL (1:1,000, AF154, R&D Systems); p-AXL (1:1,000, D12B2, Cell Signaling Technology), SRC (1:1,000, 32G6, Cell Signaling Technology), p-SRC (1:1,000, CAT#:2105, Cell Signaling Technology); S100A10 (1:1,000, 4E7E10, Santa Cruz Biotechnology), ANXA2 (1:1,000, CAT#: NBP1-31310, Novus Biologicals), and HSP70 (1:1,000, H5147, Sigma Aldrich). Secondary antibodies used in this study were HRP-conjugated anti-goat (1:50,000; sc-2020, Santa Cruz Biotechnology), HRP-conjugated anti-mouse (1:5,000; 7067P2, Cell Signaling Technology), or HRP-conjugated anti-rabbit (1:5,000; 7074P2, Cell Signaling Technology). Immunoblots were developed with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and visualized with ChemiDoc XRS+ imaging system equipped with Image Lab Software (Bio-Rad Laboratories).

For GAS6 stimulation, cells were serum deprived for 48 hours. Cells were then pretreated with PP2 (500 nmol/L) or dasatinib (50 nmol/L) for 2 hours followed by addition of GAS6 (200 ng/mL) for 4 hours.

In vivo Matrigel plug assay

All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee of Stanford University in accordance with institutional and NIH guidelines. Six to 8-week-old Rag2−/−Il2rg−/− double knockout mice were used for the in vivo plug assay. Growth factor–reduced Matrigel (250 μL) was mixed with 250 μL of conditioned media (CM) from ccRCC cells described above and subcutaneously injected into the mice. After 10 days, Matrigel plugs were analyzed for angiogenesis by macroscopic evaluation, histologic analysis, and quantified by analyzing hemoglobin content with Drabkin reagent (Sigma-Aldrich). For hemoglobin analysis, Matrigel plugs were dissected by an Eppendorf tube containing 0.5 mL of deionized water. A 10-fold dilution of Matrigel was subjected to an Eppendorf tube containing 0.5 mL of deionized water. After overnight incubation at 37°C, lysates were centrifuged at 12,000 × g for 10 minutes to remove tissue pieces and gel. The supernatant was used for hemoglobin quantification with Drabkin assay according to the manufacturer’s instructions. The final result was normalized to the initial Matrigel weight.

Tumor xenografts

To establish orthotopic ccRCC xenograft model, Rag2−/−Il2rg−/− double knockout mice aged from 6 and 12 weeks were injected with a 100 μL of collagen plug (BD Biosciences) containing 1 × 106 ccRCC cells under the renal capsule as previously described (21). For more details, please see Supplementary Methods.

PDXs

All patients who participated in this study provided written informed consent for collection and research use of their materials and use of these samples was approved by the Stanford University Institutional Review Board (IRB #34175) in accordance with recognized ethical guidelines per the U.S. Common Rule. Patient derived tissue RCC054 was obtained from a 44-year-old male undergoing surgery to remove metastatic RCC tissue from the colon at Stanford under an institutional review board–approved protocol with informed consent. We established two additional RCC PDX models from materials received from the NCI Patient-Derived Models Repository (PDMR), NCI-Frederick, Frederick National Laboratory for Cancer Research, Frederick, MD. URL – https://pdmr.cancer.gov/. For more details, please see Supplementary Methods.

Drug administration

Pazopanib (LC Laboratories) was prepared in 0.5% hydroxypropylmethyl cellulose and 0.1% Tween-80. Axitinib (LC Laboratories) was prepared in 0.5% carboxymethyl cellulose (PH 2.5). Cabozantinib (LC Laboratories) was formulated in sterile saline/10 mmol/L HCl. Mice were administered 0.1 mL of pazopanib (30 mg/kg/day for RCC054, NC1597326, and NC1961994), axitinib (36 mg/kg/day for NC1961994), cabozantinib (10 mg/kg/day for RCC054 and M62), or vehicle by oral garage. For sAXL experiments, mice were treated with control (saline) or sAXL therapy at 20 mg/kg every two days by intraperitoneal injection.

RNA-seq and data analyses

RNA samples were extracted from 1 × 106 cancer cells using Qiagen RNeasy Mini Kit. Two micrograms/sample was delivered for sequencing. Briefly, cDNA was sonicated and subjected to Illumina TruSeq RNA Library Preparation Kit. Total RNA from 786-O-shAXL1, 786-O-shAXL1, 786-O-shAXL1, M62-shSCM1, and M62-shAXL1 cells were used for the preparation of RNA-seq libraries with Illumina’s TruSeq RNA Library Prep Kit v2 according to the manufacturer’s protocol. Sequencing was performed on Illumina HiSeq 2000. For more details, please see Supplementary Methods.

Statistical analysis

Continuous outcomes were analyzed by a t test when comparing two groups. Continuous outcomes were analyzed in an ANOVA model when comparing more than two groups. Pairwise post hoc testing was done using a Tukey adjustment for multiple comparisons. Repeated measures outcomes were analyzed in a mixed effects model to account for the within mouse correlations. Time to event outcomes were summarized using Kaplan–Meier curves and groups were compared using log-rank tests. The rate of plasmin generation in Fig. 4A–D were modeled using a four-parameter dose–response curve. The four-parameter dose–response curves and the t tests were performed in Prism v 8.0.1 (GraphPad Software Inc.). All other analyses were performed in SAS v9.4 (SAS Institute Inc.). All tests were two-sided with an alpha of 0.05.

Results

Genetic inhibition of AXL in ccRCC cells reduces tumor vessel density and growth

To investigate the role of AXL in regulating the angiogenic potential of ccRCC cells in vivo, we first utilized a genetic approach to knock down AXL expression in two VHL-deficient ccRCC cell lines (786-O and M62; ref. 16). Endogenous AXL expression in both ccRCC cell lines was significantly repressed by the AXL shRNA targeting sequences relative to the control shRNA.
sequences (Supplementary Fig. S1A–S1B; refs. 11, 22). No significant differences in tumor cell proliferation or survival were observed when comparing the growth of AXL knockdown or control cell lines under normoxic or hypoxic conditions in vitro (Supplementary Fig. S1C–S1H). In contrast, primary ccRCC tumor growth under the renal capsule was significantly impaired in mice injected with AXL knockdown tumor cells compared to mice injected with AXL wild-type shSCM cells (Fig. 1A–D). Both histologic analysis and quantification of CD31+ blood vessels revealed that tumor vessel density was significantly reduced in AXL knockdown tumors compared with AXL wild-type ccRCC tumors (Fig. 1E and F). These findings demonstrate that AXL is an important factor governing ccRCC tumor vascular density and growth at the kidney, suggesting a role for AXL in mediating the angiogenic potential of ccRCC cells.

AXL promotes S100A10 expression in ccRCC cells through SRC family kinase activity

To identify proangiogenic programs regulated by AXL in ccRCC cells, we performed RNA-seq based gene expression profiling of two independent AXL wild-type and AXL knockdown ccRCC cells. Direct pairwise comparison of AXL wild-type and AXL knockdown cells revealed hundreds of genes whose expression was changed more than 2-fold in AXL knockdown cells compared with AXL wild-type cells (Fig. 2A; Supplementary Table S1–S2). Forty-two genes were significantly altered (upregulated or downregulated) in both the 786-O and M62 cell lines (Fig. 2A; Supplementary Table S3). Of the 42 genes, 10 genes were significantly decreased upon AXL knockdown (Fig. 2B; Supplementary Table S4). Notably, the expression of the plasminogen receptor S100A10 was reduced greater than 2-fold in shAXL 786-O and M62 cells compared with shSCM 786-O and M62 cells (Fig. 2B). The S100A10-Annexin II plasminogen receptor complex has been shown to enhance the formation of plasmin to promote ECM degradation, neovascularization, invasion, metastasis, and drug resistance (23–25). In addition to S100A10, Annexin A2 (ANXA2) expression was reduced >1.7 fold in both AXL knockdown cell lines compared with control lines (Fig. 2B). In human ccRCC samples, AXL expression correlates with S100A10 and increased S100A10 expression independently correlates with poor patient outcomes.
survival in patients with ccRCC (Fig. 2C and D). In addition, AXL expression correlates with ANXA2 and increased ANXA2 expression independently correlates with poor patient survival in patients with ccRCC, indicating that the S100A10/ANXA2 plasminogen pathway may contribute to ccRCC tumor progression (Fig. 2E and F).

We next confirmed AXL-dependent regulation of S100A10 and ANXA2 at the mRNA and protein level in 786-O and M62 ccRCC cells. Genetic inactivation of AXL significantly reduced S100A10 mRNA and protein as determined by real-time PCR and Western blot analyses, respectively (Fig. 2G–I; Supplementary Fig. S2A–S2C). Although AXL inactivation reduced ANXA2 at the mRNA...
AXL and S100A10 promote ccRCC plasmin production and promote angiogenic processes in vitro and in vivo

Our findings above identify AXL signaling in the regulation of S100A10 expression. Normal cells and cancer cells utilize S100A10 to convert plasminogen to plasmin at the cell surface (26). Therefore, we compared the rates of plasmin generation between AXL-deficient and AXL wild-type ccRCC cells. Using an in vitro assay that measures the conversion of plasminogen to plasmin, we found that the rate of plasmin generation was significantly reduced in shAXL knockdown ccRCC (786-O and M62 cells) compared to shSCM cells (Supplementary Fig. S3A–S3B). Similarly, we utilized a siRNA approach to directly compare plasminogen conversion between AXL and S100A10 knockdown cells (Fig. 3A and B). Compared with the siControl-treated cells, both siAXL and siS100A10 knockdown cells exhibited reduced rates of plasmin production (Fig. 3C and D). These findings demonstrate that both AXL and S100A10 in ccRCC cells promote plasmin production from plasminogen.

Plasmin production can facilitate angiogenesis through multiple mechanisms. Plasmin is an enzyme that activates matrix metalloproteinases to degrade fibrin, fibronectin, laminin, and collagen within the extracellular matrix (ECM). The proteolytic breakdown of the ECM facilitates endothelial cell invasion as well as releases proangiogenic factors sequestered within the matrix (27–29). We examined whether the conditioned media from siAXL or siS100A10 ccRCC cultures affected endothelial (HUVEC) invasion compared with siControl conditioned media. HUVEC invasion through Matrigel, an extracellular basement membrane matrix, primed with conditioned media from siAXL or siS100A10-treated ccRCC tumor cells was significantly reduced compared with Matrigel inserts primed with siControl ccRCC media (Fig. 3E and F). These findings demonstrate that secreted factors regulated by AXL and S100A10 in ccRCC cells are sufficient to prime Matrigel matrix substrates to enhance endothelial cell invasion in vitro.

We next investigated the role of AXL and S100A10 in regulating the angiogenic potential of ccRCC conditioned media in vivo using a Matrigel plug assay (30). Rag2−/−Il2rg−/− mice were injected subcutaneously with Matrigel containing supernatant from siControl, siAXL, or siS100A10-treated ccRCC cells. After 10 days, Matrigel plugs were harvested and hemoglobin concentration was quantified. The hemoglobin concentration of Matrigel plugs containing siAXL and siS100A10 conditioned media was reduced (5-fold) compared with Matrigel plugs containing siControl conditioned media (Fig. 3G and H). These findings demonstrate that AXL and S100A10 promote the angiogenic potential of ccRCC cells in vivo.

To determine whether S100A10 is a key factor promoting the angiogenic potential of ccRCC cells mediated by AXL signaling. For this purpose, S100A10 expression was rescued with an exogenous expression vector in AXL knockdown ccRCC cells (Fig. 3I). The addition of S100A10 expression restored plasminogen to plasmin conversion, HUVEC invasion, and angiogenesis in the Matrigel plug assay of shAXL ccRCC cells to levels that were comparable with the shSCM control cells, indicating that S100A10 is an important factor driving the angiogenic potential of ccRCC cells downstream of AXL signaling (Fig. 3J–L).

Therapeutic inhibition of GAS6/AXL signaling inhibits ccRCC tumor growth and synergizes with pazopanib in ccRCC PDXs

The results above identify an important role for AXL signaling in mediating ccRCC tumor growth and angiogenesis, raising the intriguing possibility that AXL inhibitors may be effective in blocking ccRCC tumor progression at multiple tissue sites when utilized alone or in combination with antiangiogenic agents. In support of this notion, cabozantinib, a small-molecule TKI that targets VEGFR, MET, and AXL has shown significant responses in kidney cancer (31). Cabozantinib is approved by the FDA and the European Medicines Agency (EMA) as a first-line therapy in patients with advanced RCC. We first evaluated the efficacy of cabozantinib in ccRCC tumor cell xenografts. Cabozantinib treatment (10 mg/kg) significantly reduced the tumor growth of the ccRCC M62 tumor xenografts (Supplementary Fig. S4). These findings are consistent with previous reports of cabozantinib reducing tumor growth and angiogenesis (14, 32). We next sought to determine if selective inhibition of AXL signaling is sufficient to inhibit tumor growth and angiogenesis. For this purpose, we have developed a highly potent, selective and safe GAS6/AXL inhibitor by generating a soluble AXL decoy receptor that is fused to human IgG1 (sAXL; ref. 19). To determine the efficacy of sAXL in ccRCC tumor progression, we treated mice with established 786-O or M62 tumors under the renal capsule or liver with a common ccRCC metastatic site. Soluble AXL treatment (20 mg/kg, every two days) resulted in a significant reduction of tumor growth at both the liver and kidney compared to the vehicle treatment (Fig. 4A–D). Reduced tumor growth in the sAXL-treated ccRCC xenografts in the kidney and liver was associated with a reduction in tumor vessel density (Fig. 4E and F).

We next evaluated the efficacy of AXL-targeting agents in ccRCC PDXs. We established a PDX line (RCC054) from a ccRCC patient with colon metastases. This patient, a 44-year-old male, received radical nephrectomy surgery upon initial diagnosis. Three months after surgery, the patient developed lung metastases and was treated with pazopanib. The patient progressed on pazopanib and one year later developed colon metastases (Supplementary Fig. S5A). Tissue was collected from the metastatic colon lesion and tissue slice grafts and PDX lines were generated using our previously established methods (21). Histologic analysis of the patient tumor demonstrated that histologic features of ccRCC as well as expression of ccRCC markers including KIT, CAIX, CD10, and Pax8 (Fig. 5A; ref. 33). Moreover, histologic and IHC analysis of the PDX tumor showed that the PDX tumor maintained the ccRCC phenotype and expressed AXL as well as...
Figure 3.
AXL and S100A10 promote the angiogenic potential of ccRCC cells in vitro and in vivo. A and B, Western blot analysis of AXL and S100A10 expression in 786-O (A) and M62 (B) cells transfected with siCON, siAXL, or siS100A10 siRNA pools. HSP70 was used as a loading control. C and D, The rate of plasmin generation in 786-O (C) or M62 (D) cells transfected with siCON, siAXL, or siS100A10. Solid lines, the average; dashed lines, the variance. E and F, Representative pictures (left) and the invasion ratio (right) of HUVEC cells invaded through Matrigel-coated membranes primed with cell culture supernatants derived from 786-O (E) and M62 (F) cells transfected with siCON, siAXL, or siS100A10. (Continued on the following page.)
S100A10 (Fig. 5A). Sequencing analysis of the PDX tissue revealed a deletion within VHL at position 492 (Fig. 5B). Pazopanib treatment did not significantly affect tumor progression in this PDX line (RCC054) when treatment began either early (7 days after tissue implantation) or with established disease (14 days postimplantation; Fig. 5C–G). In contrast, cabozantinib treatment (10 mg/kg) significantly reduced the growth of RCC054 (Fig. 5C). Moreover, single-agent sAXL therapy as well as the sAXL and pazopanib combination therapy significantly reduced the growth of RCC054 when treatment began at the early stage at 7 days following tumor implantation (Fig. 5D). The decrease in tumor growth mediated by sAXL therapy and the sAXL + pazopanib therapy was associated with a decrease in tumor vessel density (Fig. 5E). Importantly, sAXL therapy synergized with pazopanib when used to treat established disease in multiple VHL-deficient ccRCC PDX models (Fig. 5F–H; Supplementary Fig. S5B–S5F). We also observed that sAXL therapy synergized with the TKI axitinib, in ccRCC PDX NC961994 (Fig. 5H). These findings demonstrate that AXL inhibition is sufficient to inhibit ccRCC tumor progression and vessel density in ccRCC tumor xenografts and PDX models.

Discussion

Our studies reveal a role for the receptor tyrosine kinase AXL in promoting the angiogenic potential and growth of ccRCC cells. We demonstrate that genetic and therapeutic inactivation of AXL in ccRCC cells is sufficient to reduce tumor vessel density and growth at the kidney. AXL-mediated tumor growth is not associated with an intrinsic regulation of tumor proliferation or survival but rather regulation of the angiogenic potential of ccRCC cells. Previous studies linking AXL to angiogenesis have shown that AXL expression in endothelial cells promotes proangiogenic processes including endothelial migration, proliferation and tube

(Continued) G and H, Macroscopic pictures (top left), hematoxylin and eosin (H&E) staining (bottom left), and quantification of hemoglobin (right) in Matrigel plugs taken from mice 10 days following subcutaneous injection of Matrigel mixed with cell culture supernatant derived from 786-O (G) and M62 (H) cells transfected with siCON, siAXL or siS100A10. Results represent the ratio of OD540 to the weight of plus used for hemoglobin quantification. I, Western blot analysis of S100A10 expression in cell 786-O-shSCM and shAXL cells transfected with empty vector or S100A10 overexpression vector. HS770 was used as a loading control. J, The rate of plasmin generation in 786-O-shSCM and shAXL cells transfected with empty vector or S100A10 overexpression vector. K, Representative pictures (left) and the invasion ratio (right) of HUVEC cells invaded through Matrigel-coated membranes primed with cell culture supernatants derived from 786-O-shSCM and shAXL cells transfected with empty vector or S100A10 expression vector. L, Macroscopic pictures (top left), hematoxylin and eosin staining (bottom left) of the Matrigel plugs, and quantification of the hemoglobin (right) in Matrigel plugs taken from mice 10 days following subcutaneous injection of Matrigel mixed with cell culture supernatant derived from 786-O-shSCM and shAXL cells transfected with empty vector or S100A10 expression vector. Data represent the averages ± SD. *, P < 0.05; ***, P < 0.01.
formation (34–37). One mechanism by which AXL signaling on endothelial cells promotes proangiogenic functions is through the regulation of VEGFA-mediated PI3K/Akt signaling (38). Our study identifies an important role for AXL signaling in ccRCC tumor epithelial cells in the regulation of angiogenic processes including endothelial cell invasion.

We define a pathway by which GAS6/AXL signaling promotes the angiogenic potential of ccRCC cells through the activation of the S100A10/plasmin pathway. The plasminogen receptor S100A10 plays an important role in plasmin production by endothelial cells, macrophages and cancer cells. S100A10-deficient mice exhibit reduced fibrinolysis in response to batroxobin-induced vascular thrombi and have reduced macrophage recruitment in response to inflammatory stimuli (24, 39). Moreover, S100A10-deficient endothelial cells have impaired neovascularization in Matrigel plugs in vivo, associated with reduced tumor

Figure 5. AXL inhibitors synergize with antiangiogenic therapy to inhibit ccRCC PDX tumor growth. A, Hematoxylin and eosin (H&E) and IHC staining of CAIX, Ku70, CD10, CD117, AXL, and S100A10 within the primary patient (top) and PDX (bottom) tissue. B, Sequencing of VHL in RCC054-PDX tumor showing a VHL deletion mutation at nucleotide 492. C, Total tumor weights of RCC054 PDX treated with pazopanib (PA) or cabozantinib. D, Schematic description of sAXL and pazopanib for early RCC054-PDX treatment scheduling (top) and tumor weight (bottom). E, CD31 staining (top) and blood vessel number (bottom) of PDX tumors taken from mice treated with sAXL, pazopanib, or combo (sAXL + PA; three mice, 10 fields per tumor). F, Schematic description of sAXL and pazopanib for established RCC054-PDX treatment scheduling (top) and tumor growth curve (bottom). G, Total tumor weights of RCC054-established study in F at the endpoint analysis. H, Schematic description of sAXL, axitinib (AX), and pazopanib for established NCI961994-PDX treatment scheduling (top) and tumor weight (bottom). Data represent the averages ±SD. *P < 0.05, **P < 0.01; ***P < 0.001.
growth and vascular density, suggesting a role for S100A10 in angiogenesis (24, 40). In cancer cells, S100A10 expression is increased relative to normal tissue where it promotes tumor migration, invasion and metastasis (for a recent review; ref. 41). S100A10 gene expression can be induced by signaling events mediated through IFNγ, glucocorticoids, TGFβ, gonadotropin, EGF, bFGF, and IL1B. In addition, ANXA2 has been shown to regulate S100A10 mRNA and protein stability (for a recent review; ref. 26). Our study demonstrates that S100A10 expression can be induced by GAS6/AXL signaling in an SRC-dependent manner to promote the angiogenic and invasive potential of ccRCC cells. Previous reports have shown that S100A10 is increased in renal cell carcinoma relative to normal tissue (42, 43). We demonstrate that elevated S100A10 correlates with poor survival in patients with ccRCC, suggesting S100A10 may be an important factor in the pathogenesis of ccRCC. Future studies are needed to define the role of S100A10 in ccRCC tumor growth, metastasis and TKI resistance.

Our study has important therapeutic implications for the treatment of advanced ccRCC. Kidney cancer is among the top 10 most common cancers in men worldwide (1). Although antiangiogenic therapy has increased disease-free progression and survival in patients with advanced kidney cancer, the majority of patients eventually progress and succumb to metastatic disease (44). Mechanisms of angiogenic resistance include activation of compensatory angiogenesis pathways as well as increased tumor invasion (6, 7). Our data demonstrate that AXL promotes the angiogenic potential of ccRCC cells in vitro and in vivo. Moreover, we found that treatment with the AXL targeting agents cabaconibin or soluble AXL decoy therapy can reduce tumor growth and vascularization in ccRCC tumor xenografts and PDX models. We report that selective inhibition of AXL using the sAXL decoy receptor was sufficient to reduce tumor growth as a single-agent therapy and synergize with pazopanib or axitinib in ccRCC PDX models. Our data are supported by a recent publication demonstrating that AXL expression is increased in sunitinib-resistant patients with ccRCC (14). Overall, our data suggest that anti-AXL therapy may be an effective and safe strategy to prevent and treat pazopanib-resistant disease and provide preclinical data to support the rationale combination of AXL inhibitors with antiangiogenic agents for the treatment of advanced kidney cancer.

Disclosure of Potential Conflicts of Interest

Y.R. Miao has ownership interest (including patents) in Aravive Biologics. Inc. A.J. Giaccia has ownership interest (including patents) in Aravive. E.B. Rankin has ownership interest (including patents) in Pateni (US2011/022125 and US Patent (US2013/074766). No potential conflicts were disclosed by the other authors.

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