Experimental Cell Research 355 (2017) 47–56

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Gas6-Axl signaling in presence of Sunitinib is enhanced, diversified and sustained in renal tumor cells, resulting in tumor-progressive advantages

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ARTICLE INFO

Keywords:
Renal cancer
Tumor promotion
Tumor progression
Receptor tyrosine kinases
Drug resistance
Novel mechanism

ABSTRACT

Clear Cell Renal Cell Carcinoma (CCRCC) is a lethal cancer with bad prognosis due to development of chemoresistance and recurrence of more aggressive tumors. Investigation of Gas6-mediated Axl signaling in CCRCC and endothelial cells reveals a Sunitinib resistant Gas6-Axl signaling that is sustained and enhanced and specifically triggers downstream AKT and PRAS40 activation in an intensified manner. Gas6-induced Axl signaling in presence of Sunitinib is also diversified displaying onset of Axl-dependent EGFR and METR activation and activation of classical MAPK pathways. Gas6+Sunitinib-adapted CCRCC cells present increased viability and decreased apoptosis and enhanced production of the multi-tumorigenic Osteopontin (OPN) and of one of its activator matrix metalloproteinase-7. Axl activity is necessary for CCRCC cell sphere formation and the ability of the cells to attach after non-adhesive growth. In addition, Gas6+Sunitinib-adapted CCRCC cells displayed enhanced migration and sphere formation, both mechanisms being Axl and OPN dependent. Altogether, this suggests that Sunitinib while targeting endothelial cells and tumor angiogenesis, simultaneously provides protumorigenic effects due to a constitutively, intensified and divergent Gas6-Axl system.

Implications: Gas6-mediated Axl signaling, which is enhanced and diversified in the presence of Sunitinib possibly contributes to acquired chemoresistance, recurrence of aggressive disease and metastasis of CCRCC tumors. Therefore, combinatorial Axl-targeted therapy might be beneficial for CCRCC patients intended for Sunitinib treatment.

1. Introduction

Axl is a receptor tyrosine kinase (RTK) that is a member of the TAM RTK family comprising Tyro3, Axl and MerTK, which has important functions in vascular biology, immunology and tumorigenesis [1,2]. Axl affects tumor growth, epithelial-to-mesenchymal transition (EMT), angiogenesis, metastasis and development of chemoresistance to targeted therapy. Axl overexpression is linked to adverse outcome and poor survival in many cancers and Axl inhibition in several cancer models results in decreased tumorigenesis [3]. The Axl-ligand Gas6 is a vitamin K-dependent protein and contains a γ-carboxyglutamic acid-rich domain that binds calcium and negatively charged phospholipids. The γ-carboxylation is shown to be necessary for proper Gas6 function [4]. Gas6-ligation of Axl drives conventional receptor dimerization and subsequent activation of conserved signaling pathways [5]. Axl is also a target for atypical ligand-independent activation, such as crosstalk between Axl and VEGFR, EGFR and HGFR signaling, detected in cancers. These novel crosstalk mechanisms broaden the repertoire of Gas6-Axl signaling [6].

Renal Cell Carcinoma (RCC) is a lethal cancer with bad prognosis due to development of chemoresistance and recurrence of an aggressive tumor with increased tumor-angiogenesis and metastasis [7]. We have found Axl and Gas6 to be differentially presented in RCC subtypes, and Axl to correlate to tumor advancement, and to add prognostic information to patient survival [8]. Inhibition of tumor-angiogenesis by targeting of VEGFRs and PDGFRs on endothelial cells (EC) by the small molecule inhibitor Sunitinib is first-line treatment of advanced clear cell RCC (CCRCC). However, life improvement of treated patients is poor, and there is need for advancement of treatment options [7,9]. As mentioned, Axl regulates multiple tumorigenic processes contributing to advanced disease. For instance, in tumor-angiogenesis the Gas6-Axl pathway governs EC migration and recruitment and sprouting of new vessels [10]. Axl is also involved in EMT, a cellular event rendering epithelial cells more mesenchymal-like and motile, and by this means Axl plays an important role in the metastatic process [11–13]. Administration of soluble Axl in circulation prevents metastasis of cancer cells, and knockdown of Axl in CCRCC tumor cells results in lower metastatic events [11,12,14].

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http://dx.doi.org/10.1016/j.yexcr.2017.03.040
Received 9 November 2016; Received in revised form 26 February 2017; Accepted 18 March 2017
Available online 19 March 2017
0014-4827/ © 2017 Published by Elsevier Inc.

Moreover, Axl is suggested to mediate achieved resistance in several cancers, for instance by "taking-over" tumor promoting signaling from targeted RTKs, or by diversification of targeted signaling pathways [15–17]. Axl is also involved in maintaining stemness and support of non-adhesive growth of cancer cells [11,18], mechanisms strongly related to non-solid tumor cell survival, spread and repopulation of secondary organs [19]. Today, several Axl-specific inhibitors are under development. R428 is one with high Axl-selectivity and treatment with R428 inhibits tumor-angiogenesis and blocks tumor spread *in vivo* [20].

With this knowledge, we hypothesized that there is a connection between recurrence of tumor-angiogenesis and metastasis in resistant CCRCC patients and activity of the Gas6-Axl system. We now show that the Gas6-Axl system can be constitutively activated in CCRCC cells, with sustained signaling and activation of pathways known to mediate tumor advantage. Sunitinib does not inhibit but rather increases and diversifies Gas6-Axl activation and downstream signaling in CCRCC and EC cells, which results in Axl-dependent differential outcomes with enhanced migration, sphere-formation, ability to repopulate after non-adhesive growth and secretion of oncogenic proteins such as the multi tumorigenic Osteopontin (OPN) and one of its activators, the matrix metalloproteinase-7 (MMP7). Altogether, this forms a feasible self-sufficient tumor-promoting system with unresponsiveness to current Sunitinib treatment followed by enhancement of disease making Axl a highly interesting drug target in CCRCC.

2. Material & methods

2.1. Cell culture

786-O cells (ATCC) were cultured in DMEM with 10% FBS, 1% L-glutamine and 1% Penicillin/Streptomycin (Gibco/Thermo Fisher). Similar media with 0.1% FBS were used as 786-O starvation media. HAECs (Invitrogen) were cultured on 0.1% Gelatin in Cascade Biologics Medium 200 with Cascade Biologics Low Serum Growth Supplement (Gibco/Thermo Fisher). Similar media with no FBS were used as HAEC starvation media.

2.2. Gas6 expression and purification

Recombinant human Gas6 was expressed using HEK293 cells in 10 μg/mL Vitamin K as described [21]. Gas6 containing fractions were pooled and stored in aliquots at −70 °C after verification of protein purity.

2.3. Immunoblotting using Western Blot

Cells were harvested on ice in lysis buffer (25 mM Tris pH 7.5, 150 nM NaCl, 5 mM EDTA, 1% Triton X-100, 10% Glycerol) with HALT Protease Inhibitor Cocktail (Pierce/Thermo Fisher) and 1 mM sodium orthovanadate. Cells were detached by scraping. Cellular debris was removed by pelleting at 10 000 g, 5 min, 4 °C. Protein content in total cell lysate (TCL) was evaluated using BCA assay kit (Pierce/Thermo Fisher), and 5 μg TCL was separated on 4–15% TGX SDS-PAGE gel (Bio-Rad Laboratories) under reducing conditions. Proteins were blotted onto a PVDF membrane using Trans-Blot Turbo (Bio-Rad Laboratories). Primary immunoblotting (IB) antibodies were Phospho-Tyrosine monoclonal (pY99, Santa Cruz) diluted 1:5000, Axl-intracellular domain (ICD) polyclonal affinity purified (H124, Santa Cruz) diluted 1:1000, Phospho-Ser473AKT, Phospho-Thr308AKT, AKT-pan, Phospho-Thr246PRAS40, Phospho-Thr202-185/Tyr204-187 ERK1-2 (R & D Systems) reconstituted to 0.2 mg/mL and diluted 1:1000, ERK1-2, Phospho-Thr180/Tyr182P38MAPK, P38MAPK, phospho-Thr183/Tyr185 SAPK-JNK, SLUG, OPN polyclonal (R & D Systems) affinity purified reconstituted to 0.2 mg/mL and diluted 1:1000, MMP7 (1) polyclonal affinity purified (Abcam) used at 0.1 μg/mL, MMP7 (2) monoclonal (R & D Systems) reconstituted to 0.5 mg/mL and diluted 1:1000, MMP9 (G657) polyclonal affinity purified, MMP3, β-ACTIN (Sigma-Aldrich) diluted 1:100 000, and GAPDH. All primary antibodies are monoclonal antibodies from Cell Signaling diluted 1:1000 if not stated otherwise. Detecting HRP-conjugated secondary antibodies were swine anti-rabbit, goat anti-mouse, and rabbit anti-goat, all polyclonal antibodies from DAKO diluted 1:2000. Signals were developed using Immobilon ECL reagent (Millipore) and detected with a CCD camera (Bio-Rad Laboratories). For reprobing, bound antibodies were removed by 0.4 M NaOH before new IB. Band intensities were quantified and normalized to total protein using ImageLab software (Bio-Rad Laboratories). Program settings ensured no signal saturation. Expression relative housekeeping is adjusted to mean intensity of B-ACTIN & GAPDH.

2.4. Immunoprecipitation of Axl, OPN and MMP7

100 μg protein diluted in 500 uL lysis buffer were used for immunoprecipitation (IP). Samples were pre-cleared with Protein-G Sepharose (Invitrogen). IP was allowed during 1.5 h at 4 °C with rotation and pelleted with Protein-G Sepharose. IP slurry and remaining supernatant (IP supernatant) were analyzed using SDS-PAGE and IB as described with the exception that OPN polyclonal antibody (Abcam) was used for OPN IB detection. Axl-extracellular domain (ECD) and OPN polyclonal affinity purified and MMP7 monoclonal antibodies (2 μg; R & D Systems) were used for IP.

2.5. Short- and long-term cell stimulation experiments

Cell number seeded was optimized for each experiment with respect to preferred cell density at time of and during stimulation (subconfluent and confluent). *Short-term*; cells were recovered in complete media before starvation and stimulation in starve media. *Long-term*; cells were directly seeded in complete stimulation media. If starved, 786-O cells were incubated in starvation media for about 18–20 h before exchange to stimulation media. In HAECs, starvation time was reduced to 2 h. *Adapted cells (14-day)*; Cells were treated as described for long-term culture. Then media was changed at day 3 and cells were passaged at day 6 and 9 into fresh stimulation media and again media was changed at day 11. Cells were harvested and analyzed at day 14. Reagents and final concentration used for different stimulation experiments were if not stated otherwise recombinant human 200 ng/mL Gas6, 0.1 μM Sunitinib (SYNKINASE) and 1 μM R428 (SYNKINASE).

2.6. Highlights short-term Gas6+Sunitinib experiment

Confluent starved cells were pre-incubated with Sunitinib and R428 in starve media for 2 h (regarded as starvation time for HAECs), before exchange to similar media supplemented with Gas6. After 15 min Gas6-stimulation was terminated on ice and subjected to cell lysis.

2.7. Phospho-Kinase, phospho-RTK and Oncology array

Human phospho-Kinase array, phospho-RTK array and XL Oncology array (R & D Systems) were used as described by manufacturer using 150 μg of TCL (phospho-kinase and phospho-RTK array) or 150 μg of conditioned cell media (XL Oncology array). Dot intensities were quantified and normalized to control dots on each membrane supplied in triplicates using ImageLab software (Bio-Rad Laboratories). Program settings ensure no development beyond signal saturation.

2.8. Viability and apoptosis assay

786-O cells were seeded directly in stimulation media in triplicates to be either subconfluent during experiment (100 cells/well) or to be confluent at time of stimulation (10 000 cells/well). At day 6 cells were

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