Apoptotic Bodies Elicit Gas6-Mediated Migration of AXL-Expressing Tumor Cells


Abstract

Metastases are a major cause of cancer mortality. AXL, a receptor tyrosine kinase aberrantly expressed in many tumors, is a potent oncogenic driver of metastatic cell motility and has been identified as broadly relevant in cancer drug resistance. Despite its frequent association with changes in cancer phenotypes, the precise mechanism leading to AXL activation is incompletely understood. In addition to its ligand growth arrest specific-6 (Gas6), activation of AXL requires the lipid moiety phosphatidylserine (PS). Phosphatidylserine is only available to mediate AXL activation when it is externalized on cell membranes, an event that occurs during certain physiologic processes such as apoptosis. Here, it is reported that exposure of cancer cells to phosphatidylserine-containing vesicles, including synthetic liposomes and apoptotic bodies, contributes to enhanced migration of tumor cells via a PS-Gas6–AXL signaling axis. These findings suggest that anticancer treatments that induce fractional cell killing enhance the motility of surviving cells in AXL-expressing tumors, which may explain the widespread role of AXL in limiting therapeutic efficacy.

Implications: This study demonstrates that motility behavior of AXL-expressing tumor cells can be elicited by Gas6-bearing apoptotic bodies generated from tumor treatment with therapeutics that produce killing of a portion of the tumor cells present but not all, hence generating potentially problematic invasive and metastatic behavior of the surviving tumor cells.

Introduction

AXL is a member of the TAM (Tyro3, AXL, MerTK) family of receptor tyrosine kinases (RTK). Under healthy conditions, TAMs serve a prominent role in regulating the innate immune system (1), but in tumor cells, their aberrant expression promotes survival, chemoresistance, and motility (2). The mechanism of TAM receptor activation is unique among RTK families, requiring both a protein ligand and the lipid moiety phosphatidylserine (PS; refs. 3, 4). In healthy cells, nearly all phosphatidylserine is present on the inner leaflet of the plasma membrane but is externalized on apoptotic cell membranes and apoptotic bodies (AB; refs. 5, 6). Phosphatidylserine exposure allows immune cells that express TAM receptors to engulf these membrane structures. At the same time, TAM activation negatively regulates the innate immune system (1, 7, 8). Consistent with these roles, TAM knockout (KO) mice exhibit accumulation of phosphatidylserine-positive cell debris in various tissues and autoimmune disorders (9, 10). The role of phosphatidylserine in driving TAM-mediated immune cell responses is well established, but the contribution of phosphatidylserine in TAM-mediated cancer signaling remains poorly understood.

In cancer, expression of AXL widely correlates with poor survival and is associated with drug resistance, migration, invasive- ness, and metastatic spread (11–14). RTKs such as EGFR have been reported to transactivate AXL in a ligand-independent manner (15), whereas ligand-dependent activation of AXL is mediated by phosphatidylserine and the bridging ligand Gas6 (16). γ-Carboxylation of the amino terminus of Gas6 is required for its interaction with phosphatidylserine, while the carboxy-terminal domain of Gas6 binds to the AXL ligand–binding domains (Fig. 1A). AXL and Gas6 interact through high-affinity (Ig1) and low-affinity (Ig2) binding interfaces (Fig. 1A). We previously reported the mechanism of this ligand-dependent AXL activation, wherein extracellular vesicles enriched in phosphatidylserine cluster Gas6 ligand, which increases local ligand concentration. This localized concentration promotes binding at the low-affinity site Ig2 of ligands already bound at the high-affinity site Ig1. In conjunction with diffusional transport of unoccupied AXL within the plasma membrane to the sites of localized Gas6 presentation, this asymmetric bivalent binding process leads to enhanced AXL activation (17). These findings motivated us to explore the phenotypic consequences of this unique phosphatidylserine-dependent mechanism of receptor activation.

ATP-dependent enzymes called flippases normally keep phosphatidylserine inside the cell, but phosphatidylserine is exposed by the activation of scramblases on the cell surface in biological processes such as apoptosis (18, 19). However,
even in nonapoptotic tumor cells, phosphatidylserine can appear on the outer leaflet, which is accompanied by increased shedding of microvesicles (20, 21). In addition, higher rates of apoptosis within the tumor environment, as well as therapy-induced apoptosis, contribute to bursts of phosphatidylserine exposure and release of phosphatidylserine-expressing apoptotic bodies.

Thus, we hypothesized that surviving AXL-expressing tumor cells may take advantage of a resulting phosphatidylserine-mediated increase in local Gas6 ligand concentration, leading to increased AXL activation and the phenotypic alterations of these cells. This may help explain the widely reported role of AXL in cancer therapeutic resistance. In this report, we investigate the effect of phosphatidylserine-expressing vesicles on cancer cell behavior. We find that PS/Gas6-dependent AXL signaling promotes cancer cell migration, but does not alter cell proliferation. Our results offer new mechanistic insights into the microenvironmental conditions under which AXL may significantly contribute to cancer cell migration, providing understanding that should help facilitate more effective therapeutic targeting.

Figure 1.
Phosphatidylserine (PS)-mediated AXL activation is important for migration. A, Gas6 binds to phosphatidylserine on extracellular vesicles, driving AXL dimerization and activation. Therefore, two strategies for inhibiting AXL activation are by preventing the Gas6–PS interaction using warfarin, or inhibiting the tyrosine kinase domain with R428. B, Phosphorylated AXL (pAXL), total AXL, and Gas6 levels quantified after 24 hours of treatment with 1 μmol/L R428 or 100 μg/mL warfarin. Data are means ± SEM of three biological replicates. All measurements are significantly different (P < 0.05, Student t test) compared with control, except for bars annotated with NS (not significant). C, Polarity-sensitive Annexin-V Green binding (22) to exposed phosphatidylserine in MDA-MB-231 (left) and SK-MES-1 (right) cells after 24 hours of culturing. A green fluorescent signal is only emitted when bound to phosphatidylserine on apoptotic cells. D, Cell proliferation measured in a CellTiter Glo assay after 72 hours of treatment with 1.25 nmol/L Gas6, 1 μmol/L R428, or 100 μg/mL warfarin. Data are means ± SEM of three biological measurements. E, Cell migration measured in a wound scratch assay after treatment with 1.25 nmol/L Gas6, 1 μmol/L R428, or 100 μg/mL warfarin. The relative wound density (RWD), a representation of the cell density (per unit area) in the established wound area relative to the cell density outside of the wound area, was measured over 24 hours. A representative graph of one experiment performed in replicates of six is shown.
Materials and Methods

**Reagents**

Gas6 was obtained from R&D Systems. Warfarin was obtained from Santa Cruz Biotechnology. R428 was obtained from Thermo Fisher Scientific. DOPS (1,2-dioleoyl-sn-glycer-3-phospho-L-serine, sodium salt), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), and Coagulant I (DOPC:DOPS:DOPC, 5:3:2 w/w) were obtained as 99% powder from Avanti Polar Lipids, Inc.

**Cell culture**

All cell lines were obtained from ATCC. Cells were tested and authenticated according to the cell banks protocol, by assessing short tandem repeat DNA profiles. Mycoplasma tests were performed in-house by using MycoAlert Mycoplasma Detection Kit (Lonza). MDA-MB-231 cells were grown in DMEM, HCC827 in RPMI1640, and SK-MES-1 in EMEM, all supplemented with 10% FBS and 1% penicillin-streptomycin. Nuclear red MDA-MB-231 and SK-MES-1 cell lines were obtained by transfection with NucLight Red Lentivirus Reagent (Esen BioScience) according to the manufacturer’s protocols.

**Generation of AXL KO cell line using CRISPR/Cas9**

An AXL KO MDA-MB-231-red cell line was generated with the CRISPR/Cas9 system, using plasmid DNA that was kindly provided by the Jaenisch laboratory (MIT, Boston, MA). The gRNA sequence TCGCCAAGATGCCAGTCAAG targeting the AXL kinase domain was subcloned in a mammalian expression CRISPR/Cas9 and GFP vector (pSpCas9(BB)-2A-GFP (PX458), Addgene #48138; ref. 22). Cells were transfected in 6-well plates using 8 μg of plasmid DNA and 20 μL LipoFectamine 2000 transfection reagent (Thermo Fisher Scientific) per well in OptiMEM (Life Technologies) for 5 hours. Forty-eight hours after transfection, cells were trypsinized, washed in PBS, and resuspended in Odyssey Blocking Buffer (LI-COR Biosciences). Cells were incubated overnight with primary AXL antibody MAB154 (R&D Systems), followed by secondary antibody anti-mouse Alexa Fluor 647. Finally, cells were sorted for the GFP-positive and AXL-negative population using a BD SORP FACSAria I cell sorter. This AXL-negative cell population was cultured for subsequent weeks, and AXL KO was confirmed using ELISA (described below).

**Detection of phosphatidylserine in cell cultures**

Cells were seeded overnight in full serum medium at a density of 5 × 10^5 cells per 96-well and then cultured in serum-free medium in the presence of IncuCyte Annexin V Green Reagent, a highly selective phosphatidylserine cyanine fluorescent dye (23). Cells were cultured for 48 hours inside an incubator imaging system (Incucyte, Essen BioScience) that allowed images to be automatically acquired and registered using a 10× objective.

**Preparation of cell lysates**

Cells were seeded sparsely (2 × 10^5 cells/well) in 6-well plates overnight and serum-starved for 4 hours in serum-free medium supplemented with 0.35% BSA. Warfarin was continuously present in the cell culture medium throughout the entire culture period to prevent Gas6 γ-carboxylation. After starvation, cells were treated with 100 μg/mL warfarin or 1 μmol/L R428 for 24 hours. Cells were lysed in 50 mmol/L Tris, 10% glycerol, 150 mmol/L NaCl, 1% NP40 at pH 7.5 with cOmplete protease inhibitor (Roche), and phosphatase inhibitor I (Boston Bioproducts). Protein concentration was measured by bicinchoninic acid assay following the manufacturer’s protocol (Thermo Fisher Scientific).

**Quantification of receptor abundance and phosphorylation**

All ELISA measurements were performed in multiplexed fashion, using individually identifiable beads (Bio-Rad Laboratories). Brieﬂy, beads were sedimented for 3 minutes at 10^4 × g and then resuspended in 80 μL of 100 mmol/L NaH_2PO_4 pH 6.3. Ten microliters of 50 mg/mL S-NHS (Invitrogen) and 10 μL of 50 mg/mL EDC were added, and the mixture was incubated with agitation for 20 minutes at room temperature. Beads were then pelleted and resuspended in 300 μL 50 mmol/L HEPES pH 7.4 with 0.1 mg/mL of either AXL, Tyro3, MerTK, or Gas6 capture antibody (R&D Systems). The mixture was incubated overnight at 4°C with agitation. The next day, the beads were washed repeatedly and stored in 1% BSA in PBS. Coupling efficiency was measured using biotinylated protein G.

Lysates were incubated with capture beads overnight at 4°C with agitation and then washed with 0.1% Tween-20 in PBS and incubated with either detection antibodies for AXL/Tyro3/MerTK/Gas6 (R&D Systems) or biotinylated anti-phosphotyrosine antibody 4G10 (Millipore Corp.) for 1 hour. After a second wash, beads were incubated with streptavidin-phycocerythrin (Bio-Rad Laboratories) for 10 minutes and then quantified using a FlexMap 3D (Lumines Kin Corp.). AXL and Gas6 abundance was quantified by comparison with a recombinant standard (R&D Systems).

**Preparation of cell debris and isolation of apoptotic bodies**

HCC827 cells were seeded in 5-cm plates at a density of 2.5 × 10^5 cells per plate and grown for 24 hours. After removal of the medium and a wash with PBS, cells were UV-irradiated (254 nm) using a dose of 5 J/cm² with an UVGL-58 handheld UV lamp (UVP). Serum-free medium (4 mL) with 0.35% BSA was added, and cell debris was collected after 24 hours. Cells were sedimented for 10 minutes at 300 × g, and the remaining cell debris mixture was used for assays. To isolate apoptotic bodies, the mixture was centrifuged for an additional 20 minutes at 16,500 × g using a Sorvall RC 6 Plus superspeed centrifuge equipped with the Sorvall SS-34 fixed-angle rotor (Thermo Fisher Scientific; ref. 24). Dynamic light scattering (DLS) measurements were performed with a DynaPro NanoStar (Wyatt Technology Corp.) to determine the distribution and size of the vesicles.

**Preparation of liposomes**

Lipids were stored as powder under argon at −20°C prior to use. Powders were hydrated with PBS to total lipid concentrations of about 20 mg/mL. Multilamellar vesicles (MLV) were formed through 5 minutes of high-speed physical agitation with a vortex mixer. Sonicated unilamellar vesicles (SUV) were subsequently prepared from MLV solution through additional pulse sonication for 10 minutes (10 seconds on/off) with a Branson SLPe probe-tip sonicator (Branson Ultrasonics) at 45% maximum power. MLV and SUV solutions were passed through sterile hydrophilic 0.45 μm CA and 0.22 μm PVDF filters, respectively. Liposomes were used in cell culture immediately following synthesis and analyzed for size and distribution by DLS.

**Cell proliferation assay**

Cell proliferation was evaluated using the CellTiter-Glo Luminiscence Cell Viability Assay (Promega). Cells were seeded...
Results

Wound confluence assay

Cells were plated at a density of 4.5 × 10⁴ cells per well in an uncoated 96-well Essen ImageLock plate, allowed to grow overnight in full serum medium at a density of 1 × 10⁵ cells per 96-well and then cultured in serum-free medium in the presence of inhibitors, liposomes, cell debris, and apoptotic bodies for 72 hours. For knockdown, cells were transfected with 25 nmol/L of nontargeting siRNA or siRNA targeting AXL using DharmaFECT 1 (SK-MES-1) or DharmaFECT 4 (MDA-MB-231) transfection reagent (GE Dharmacon), according to the manufacturer’s instructions. All further analysis was performed 72 hours after siRNA transfection.

PS-Gas6–mediated AXL activation enhances cancer cell migration

To investigate the phenotypic cellular response of AXL signaling mediated by PS-Gas6, we first identified cell lines that are strongly affected by phosphatidylserine-mediated AXL activation due to autocrine Gas6/PS production. We inhibited AXL signaling using the tyrosine kinase inhibitor R428 or warfarin (Fig. 1A). Warfarin prevents synthesis of vitamin K and thus γ-carboxylation of Gas6, thereby preventing its interaction with phosphatidylserine. R428 inhibits AXL in a ligand-independent manner, therefore perturbing the AXL signaling pathway in a different manner compared with warfarin. The use of both agents enabled us to identify the specific contribution of phosphatidylserine to AXL-mediated signaling. Examining a panel of nine triple-negative breast cancer (TNBC) and non–small cell lung cancer (NSCLC) cell lines (Supplementary Fig. S1A), the TNBC cell line MDA-MB-231 and NSCLC cell line SK-MES-1 exhibited great reliability of AXL activation on PS-Gas6–mediated signaling (Fig. 1B). AXL phosphorylation decreased upon addition of R428 as well as warfarin, the effects of the latter specifically implicating the role of autocrine phosphatidylserine-mediated signaling in AXL activation.

To confirm the presence of exposed phosphatidylserine on MDA-MB-231 and SK-MES-1 cells in culture, we performed an Annexin V binding assay (23). In this assay, a polarity-sensitive Annexin-based biosensor with switchable fluorescence states emits green fluorescence when bound to phosphatidylserine on apoptotic cells. As shown in Fig. 1C, phosphatidylserine presenting cells and vesicles were identified in the populations of both cell types, confirming the availability of this lipid to activate AXL.

Although both R428 and warfarin inhibited AXL activation, they appeared to do so by distinct effects on cellular signaling and/or trafficking processes. This was indicated by analysis of the AXL expression levels, which were increased in both cell lines upon warfarin stimulation but not upon addition of R428 (Fig. 1B). Similarly, autocrine Gas6 levels were only increased in the presence of warfarin (Fig. 1B).

The two inhibitors also produced different cell proliferation responses. Inhibition of the AXL tyrosine kinase domain by R428 decreased cell proliferation profoundly in MDA-MB-231 and SK-MES-1 cells (Fig. 1D). The role of AXL in this response was confirmed with AXL siRNA treatment, which inhibited proliferation to a similar extent as with R428 treatment in these cell lines (Supplementary Fig. S1B and S1C). On the other hand, disruption of the Gas6–PS interaction by warfarin did not affect cell proliferation, implying that phosphatidylserine-mediated, ligand-dependent AXL signaling may not contribute to cell proliferation (Fig. 1D).

In contrast to cell proliferation, AXL-mediated cell migration was inhibited to a similar extent by R428 and warfarin in both cell lines (Fig. 1E). Endogenous ligand was sufficient, as exogenous Gas6 did not increase migration rates beyond that driven by the endogenous ligand. Both cell lines did not express Tyro3 and MerTK, the two other TAM family receptors that are activated by phosphatidylserine/Gas6, as determined by ELISA. This further supports that the warfarin-induced decrease in migration resulted from PS-Gas6-AXL inhibition. In total, these data demonstrate that ligand-dependent, phosphatidylserine-mediated AXL activation contributes to the migration of MDA-MB-231 and SK-MES-1 cells, but is not required for cell proliferation.

Apoptotic cell debris induces AXL-mediated cell migration

Our data indicate that AXL-mediated cell migration is driven via Gas6- and phosphatidylserine-dependent activation. Phosphatidylserine is usually present on the inner leaflet of cell membranes, but can be exposed when cells undergo apoptosis (18). Therefore, we hypothesized that AXL signaling may be directly upregulated in an apoptotic environment through phosphatidylserine exposure. This would potentially explain previous observations of AXL-mediated, chemotheraphy-induced migration and invasion (25).

To test this hypothesis, we performed wound scratch migration assays with MDA-MB-231 cells displaying a live-cell fluorescent nuclear reporter (MDA-MB-231-red cells) in the presence of phosphatidylserine-containing cell debris. The image processing and analysis workflow used to quantify motility of cells labeled with the red nuclear reporter in the wound assay is shown in...
Supplementary Fig. S2. We induced apoptosis in the AXL-negative tumor cell line HCC827 (no detectable AXL levels by ELISA, data not shown) by UV irradiation. Cells were removed from the culture medium by centrifugation, resulting in a cell debris mixture containing the soluble secretome as well as apoptotic bodies (Fig. 2A). Treatment of MDA-MB-231-red cells with apoptotic cell debris significantly increased their migratory rate (Fig. 2B and C). This was mediated by factors resulting from apoptosis, as migration was not affected by addition of supernatant from non-UV irradiated HCC827 cells (Supplementary Fig. S3A). Treatment with R428 or warfarin inhibited the debris-induced migration in all cases, suggesting the role of AXL in this process (Fig. 2C). Nevertheless, in several experiments, there was a significant increase in migration when comparing inhibitor alone with the combination of inhibitor and cell debris. This suggests that besides phosphatidylserine/Gas6, additional factors present in cell debris mixtures may contribute to cell migration.

Apoptotic bodies induce AXL-mediated cell migration

To identify the contribution of the phosphatidylserine-presenting vesicles to cell motility within the cell debris mixture, we first isolated ABs by centrifugation (Fig. 2A). Addition of isolated ABs increased cell migration to a similar degree as the presence of total cell debris (Fig. 3A). In contrast, the soluble apoptotic secretome did not induce cell migration (Supplementary Fig. S3B), which confirms that the observed increase in cell migration within the UV-treated supernatant fraction was due to addition of apoptotic bodies. Inhibiting AXL with either R428 or warfarin decreased the AB-induced cell migration (Fig. 3A), implicating the role of AXL and phosphatidylserine in this process. Stimulating cells with ABs did not affect cell proliferation (Supplementary Fig. S3C), which is in line with the lack of effect of phosphatidylserine-mediated AXL signaling on cell proliferation as ascertained previously (Fig. 1D).

To confirm the contribution of AXL in the apoptosis-induced migration, we repeated experiments with an AXL KO MDA-MB-231-red cell line. AXL knockout was obtained using the CRISPR/Cas9 technology (22) and confirmed with ELISA (Supplementary Fig. S4A). R428 and warfarin did not affect cell migration in the MDA-MB-231-red AXL KO cell line (Fig. 3B and C), confirming that the effects of these inhibitors in all previous experiments were exerted via the AXL receptor. AXL KO reduced the WCR compared with parental MDA-MB-231-red cells (Fig. 3D), in an order of magnitude similar as for treatment with the AXL inhibitors R428 and warfarin (Fig. 3B). Next, we compared the migration of parental and AXL KO cells in the presence of apoptotic bodies.

Figure 2.
Apoptotic cell debris induces AXL-dependent cell migration. A, Preparation and isolation of apoptotic cell debris and ABs from non-AXL-expressing HCC827 lung cancer cells by means of UV irradiation and centrifugation. B, Representative images of the wound density 24 hours after scratching a confluent MDA-MB-231-red monolayer, in the absence (left) and the presence of apoptotic cell debris (right). C, WCR of MDA-MB-231-red cells upon addition of apoptotic cell debris with or without 1 μmol/L R428 or 100 μg/mL warfarin. Data of three independent experiments are presented as log2 of median, with whiskers representing the 5 to 95 percentile of at least eight measurements per condition (*, P < 0.05; **, P < 0.01; ***, P < 0.001, Student t test).
as presented in Fig. 3E. A significant decrease in migration was observed upon addition of apoptotic bodies to the AXL KO cell line compared with the parental cell line, providing evidence that AXL signaling is contributing to apoptosis-induced migration. In the AXL KO cell line, a small but significant increase of migration upon addition of ABs remained compared with control (Supplementary Fig. S4B and S4C), which suggests that additional factors are involved in this process.

**Phosphatidylserine-containing liposomes induce AXL/Gas6–mediated cell migration**

To confirm and validate the contribution of phosphatidylserine to the increased migration response, we made use of synthetic liposomes of varying size and composition (Fig. 4A). MLVs and SUVs (Fig. 4B) were synthesized with a 5:3:2 w/w blend of DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOPS, and DOPC to yield liposome populations of individual vesicles with the blended lipid composition. The 5:3:2 w/w blend approximately represented cell plasma membrane composition after scramblase activation and was therefore selected as appropriate surrogate to mimic apoptotic bodies (26). The SUV and MLV preparations had distinct average hydrodynamic diameters (Dh) that represent distinct populations comprising sizes germane to the apoptotic cell debris (Fig. 4A). Treatment of MDA-MB-231-red cells with MLV and SUV samples of the lipid blend produced significant promigratory responses with a dependence on
liposome diameter and a biphasic dose–response relationship (Fig. 4C). Consistent with our previous work (17), this biphasic behavior reflects one of the unique features of lipid-ligand–induced AXL activation and points to changes in AXL localization giving rise to the effect of the Gas6–PS interaction rather than a conformational change due to phosphatidylserine interaction itself. This behavior is schematically presented in Fig. 4D: In the absence of lipid, Gas6 will occupy the high-affinity AXL-binding domain Ig1, but the low-affinity site Ig2 remains insufficient for receptor dimerization and activation by Gas6. On the other hand, at very high concentrations of lipid, the overabundance of phosphatidylserine prevents the occurrence of high local Gas6–AXL concentrations that are required for AXL dimerization and activation. The latter only efficiently occurs for an optimal ratio of Gas6 and lipids expressing phosphatidylserine.

To parse the effects of lipid component composition, SUVs were synthesized with component compositions of DOPS alone, DOPC alone, and a 5:3:2 w/w blend of DOPE:DOPS:DOPC (lipid blend). Treatment of MDA-MB-231 cells with SUV compositional variants at a total lipid concentration of 50 μg/mL resulted in increased cell migration only when liposomes contained phosphatidylserine (Fig. 5A). The increase in cell migration with DOPS was similar to that induced by stimulation with EGF, a known promigratory stimulus for this cell line (Supplementary Fig. S5A). As with the AB mixtures, cell proliferation was not affected by any of the phosphatidylserine-containing vesicles (Supplementary Fig. S5B).

Treatment with R428 prevented the SUV-induced migration response and inhibited cellular migration under all conditions (Fig. 5A). The combination of warfarin and the SUV
compositional variants likewise abolished the increase in migration observed for liposomes containing phosphatidylserine (Fig. 5A). Nevertheless, stimulating warfarin-treated MDA-MB-231 cells with phosphatidylserine liposomes resulted in a small but significant increase in migration compared with warfarin-only treated cells. We hypothesized that this might be the result of residual γ-carboxylated Gas6 in the cell culture medium due to high autocrine Gas6 levels in this cell line (Fig. 1B). To test this, we repeated these experiments for the other cell line, SK-MES-1, which secrete substantially lower levels of autocrine Gas6 compared with MDA-MB-231 cells.

In accordance with the effects on MDA-MB-231 cells, treatment of SK-MES-1 cells with SUV compositional variants increased cell migration only when liposomes contained phosphatidylserine (Fig. 5B). Combination treatment of SUVs and AXL inhibitor R428 or warfarin eliminated the promigratory response and inhibited cellular migration under all conditions (Fig. 5B). In contrast to MDA-MB-231 cells, stimulating warfarin-treated SK-MES-1 cells with phosphatidylserine liposomes resulted in equal migration rates compared with warfarin-only treated cells, potentially due to lower autocrine Gas6 levels.

These results indicate that migration of the cell lines tested is mediated by phosphatidylserine-containing liposomes through AXL activation. The localization of the protein ligand Gas6 by phosphatidylserine in vesicle presentation, either by synthetic liposomes or apoptotic bodies, is essential for this promigratory response.

**Discussion**

This study contributes to our expanding knowledge of the important roles that extracellular vesicles play in cancer cell signaling and function. We introduce the concept of apoptosis-induced migration by demonstrating that PS-containing vesicles, including apoptotic bodies, enhance cell migration via the AXL receptor (Fig. 6). Elucidating this mechanism of AXL activation is of great importance for designing therapeutic regimens that include AXL targeting by identifying where and when AXL is active.

The involvement of AXL in cancer metastasis has been extensively reported. AXL-mediated downstream signaling events resulting in cell migration have been heavily studied (12, 27, 28), but the AXL activation mechanism responsible for this phenotype has been largely unknown. Localized Gas6 that is bound to phosphatidylserine on extracellular vesicles was previously reported to mediate ligand-dependent AXL activation (17), and the current study demonstrates that this lipid-ligand-induced AXL signaling enhances cell motility. Warfarin, clinically used as an anticoagulant (29), prevents binding of in situ-produced phosphatidylserine and has been shown to have antimetastatic effects (30, 31). Similarly, phosphatidylserine-targeting antibodies have been reported to decrease in vivo tumor growth (32). Collectively, these data suggest that the participation of the lipid component phosphatidylserine should be incorporated when investigating the role of AXL in cancer progression.

![Figure 5](image-url)

**Figure 5.** Phosphatidylserine-containing liposomes induce AXL-mediated cell migration in MDA-MB-231 cells (A) and SK-MES-1 cells (B). WCRs upon addition of DOPS alone, DOPC alone and a 5:3:2 w/w lipid blend of DOPE:DOPS:DOPC in the presence and absence of 1 µmol/L R428 or 100 µg/mL warfarin. All lipids were present at a concentration of 50 µg/mL. Data are presented as log2 of median, with whiskers representing the 5 to 95 percentile of three independent experiments performed in replicates of six (*, P < 0.05; **, P < 0.01; ***, P < 0.001, Student t test).

![Figure 6](image-url)

**Figure 6.** Model of apoptosis-induced migration upon AXL activation by the PS-Gas6 complex as presented on apoptotic bodies. Surrounding AXL-expressing tumor cells are influenced by bursts in phosphatidylserine (PS) exposure generated by surrounding apoptotic cells, leading to increased AXL activation and cell migration.
The concept of apoptosis-induced migration described in this study implies a potential selective advantage of AXL-expressing tumor cells to migrate out of the primary tumor environment and disseminate. Results from Gjerdum and colleagues provide indications of this phenomenon, having reported that matched patient metastatic lesions exhibit higher AXL levels compared with those in the primary tumor (11). In addition, it has been demonstrated that chemotherapy-induced migration and invasion of colorectal cancer cells is regulated by AXL (25).

An increasing number of studies report signaling by extracellular vesicles as a major contributor to multiple hallmarks of cancer (33). Although most of these reports focus on the uptake of cargo such as proteins, RNA, and DNA carried by these vesicles (34, 35), the work presented here provides evidence for direct signaling initiated by the lipid bilayer component phosphatidylserine. This is highly relevant to cancer biology as phosphatidylserine is abundant within the tumor microenvironment, as disrupted membrane asymmetry in tumor cells results in phosphatidylserine exposure on the outer leaflet of the cell membrane. AXL KO partly reduced apoptosis-induced cell migration; hence, we conclude that other signaling molecules present on/in apoptotic bodies can induce cancer cell migration. In addition, phosphatidylserine acts as a lipid messenger for several other receptors, which may equally be involved in this process (36, 37). Elucidation of these factors remains a subject for future studies.

In this study, we identified apoptotic bodies as extracellular vesicles responsible for initiating increased cell motility, as isolation of smaller vesicles, such as exosomes, would require additional centrifugal force (24). However, tumor-derived exosomes have been reported to express phosphatidylserine in ovarian cancer (20, 38). As exosomes fall within the size range of the SUV liposomes used in this study (Fig. 4A), we speculate that besides apoptotic bodies, tumor-derived exosomes might also be capable of inducing AXL-mediated cell migration. Irrespective of the type of extracellular vesicles, high levels of phosphatidylserine exposure have been reported to correlate with increased malignant potential of tumor cells (21).

Therapeutic killing of tumor cells results in apoptotic cells that break down into apoptotic bodies, which are highly enriched in phosphatidylserine. Apoptosis was initially regarded as a "clean and silent" mechanism of cell death aimed at eliminating cells by the immune system. Studies in both developmental and cancer biology have revealed the signaling capacities of apoptotic cells affecting neighboring cells, resulting in tissue remodeling, cell proliferation, tumor growth, angiogenesis, and drug resistance (39–43). Our study adds PS-AXL–mediated migration to the list of the paradoxical oncocgenic properties of apoptotic cells. Interestingly, our findings revealed that the PS-Gas6-AXL–mediated response does not affect cell proliferation. For that reason, one interpretation of this work is that apoptosis-mediated activation of AXL is part of a migratory cancer cell’s “fight or flight” response during therapy, in which AXL either contributes to the development of drug resistance and/or the enhancement of metastatic capability.

The mechanistic insight into AXL-mediated migration gained from this work may help to advance the design of more effective therapeutic strategies. AXL is regarded as a promising target for addressing drug resistance and metastasis, and inhibitors for all components of the AXL signaling complex have been developed so far. These modulators include small-molecule AXL kinase domain inhibitors (14, 44, 45), an AXL “decoy receptor” that binds Gas6 to inhibit its function (46), and phosphatidylserine-targeting antibodies (32). Our results indicate that drugs targeting both AXL and Gas6 inhibit cell migration in vitro. However, only the small-molecule AXL inhibitor R428 decreased cell viability, potentially through inhibition of transactivation of other RTKs (15, 47). For that reason, small-molecule AXL kinase inhibitors could provide an additional treatment benefit by inhibiting cell proliferation and drug resistance. We argue that high AXL expression levels in the primary tumor should not necessarily be the selection criteria for patients that would benefit from AXL-targeting drugs. A small subpopulation of AXL-expressing cells within the primary tumor could be sufficient to form metastases by taking advantage of an apoptotic environment produced by fractional cell killing following therapy (11). This further implies that AXL-targeting agents should be administered simultaneously or prior to other chemotherapeutics to diminish the potential for apoptosis-mediated migration through AXL signaling.

One of the open questions raised by the current study is how apoptosis-mediated AXL signaling will manifest in a tumor environment containing immune cells and cancer cells that both express AXL (48), as well as the other TAM family receptors, MerTK and Tyro3, which are also activated by phosphatidylserine and Gas6. Binding of apoptotic bodies to AXL-expressing tumor cells may not only induce migration, but also cause juxtaocrine signaling upon interaction with TAM-expressing immune cells to block immunosurveillance (2, 31, 49). The escape from immune detection may contribute to metastatic dissemination and may promote the outgrowth of drug-resistant tumor cells. The specific role of PS-Gas6-AXL–mediated signaling in the development and maintenance of drug resistance remains to be elucidated.

In summary, we have identified novel signaling capacities of extracellular vesicles presenting phosphatidylserine in the context of cancer biology. The concept of apoptosis-induced migration may apply to a wide range of solid tumors expressing AXL and provides a rationale for including AXL inhibitors in combinatorial therapies at the onset of cancer treatments to hinder metastatic disease progression.

Disclosure of Potential Conflicts of Interest
D.A. Lauffenburger is a member of the scientific advisory board at and reports receiving a commercial research grant from Merrimack Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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