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Angiogenesis, Metastasis, and the Cellular Microenvironment

Molecular Cancer Research

Autotaxin Signaling via Lysophosphatidic Acid Receptors Contributes to Vascular Endothelial Growth Factor–Induced Endothelial Cell Migration

Malgorzata M. Ptaszynska, Michael L. Pendrak, Mary L. Stracke, and David D. Roberts

Abstract

Important roles for vascular endothelial growth factor (VEGF) and autotaxin (ATX) have been established for embryonic vasculogenesis and cancer progression. We examined whether these two angiogenic factors cooperate in regulation of endothelial cell migratory responses. VEGF stimulated expression of ATX and LPA1, a receptor for the ATX enzymatic product lysophosphatidic acid (LPA), in human umbilical vein endothelial cells. Knockdown of ATX expression significantly decreased mRNA levels for the receptors LPA1, LPA2, S1P1, S1P2, S1P3, and VEGFR2 and abolished cell migration to lysophosphatidylcholine, LPA, recombinant ATX, and VEGF. Migration to sphingosylphosphorylcholine and sphinogosine-1-phosphate was also reduced in ATX knockdown cells, whereas migration to serum remained unchanged. Furthermore, ATX knockdown decreased Akt2 mRNA levels, whereas LPA treatment strongly stimulated Akt2 expression. We propose that VEGF stimulates LPA production by inducing ATX expression. VEGF also increases LPA1 signaling, which in turn increases Akt2 expression. Akt2 is strongly associated with cancer progression, cellular migration, and promotion of epithelial-mesenchymal transition. These data show a role for ATX in maintaining expression of receptors required for VEGF and lysophospholipids to accelerate angiogenesis. Because VEGF and ATX are upregulated in many cancers, the regulatory mechanism proposed in these studies could apply to cancer-related angiogenesis and cancer progression. These data further suggest that ATX could be a prognostic factor or a target for therapeutic intervention in several cancers. *Mol Cancer Res; 8(3); 309–21. ©2010 AACR.*

Introduction

Angiogenesis is the process of new blood vessel formation from preexisting vessels, which occurs physiologically during the menstrual cycle, placental growth, and wound healing. In cancer, angiogenesis leads to the extension of preexisting vascular networks into a growing mass of tumor cells, giving tumor cells access to the circulation and facilitating further tumor growth and metastasis (1). Vascular endothelial growth factor A (VEGF) is a key angiogenic factor whose biological importance is revealed by the fact that even deletion of one allele leads to embryonic lethality (2, 3) and autocrine production of VEGF is essential for endothelial function in adult mice (4). VEGF is involved in all phases of angiogenesis and is required for growth, migration, and differentiation of endothelial cells. In addition, VEGF is a potent vascular permeability factor and plays essential roles in acute and long-term regulation of cardiovascular homeostasis (5-7).

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Among the VEGF receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR) have tyrosine kinase activity and play substantial roles in the regulation of angiogenesis. VEGFR2 signaling is the primary mediator of endothelial cell proliferation and migration (8). VEGFR1 is both a positive and negative regulator of VEGFR2 signaling and acts as a positive regulator of monocyte, macrophage, and hematopoietic cell migration (9, 10). Recent work has shown that VEGF signaling is not restricted to the regulation of angiogenesis but also contributes to tumor progression (11). Treatments targeting VEGF or its signaling pathways have been clinically proven to extend survival in several cancers (12, 13). The hypertensive and prothrombotic side effects observed during treatment with antiangiogenic therapy confirm the importance of VEGF signaling pathways in maintaining vascular and platelet homeostasis. This toxicity also indicates a need for better understanding of critical VEGF-stimulated pathways and mediators to develop more effective angiogenesis inhibitors with fewer side effects (14).

Autotaxin (ATX; NPP2), discovered as an autocrine tumor motility–stimulating factor (15), is a secreted member of the nucleotide pyrophosphatase and phosphodiesterase (NPP) family of ectoenzymes/exoenzymes (16). ATX has physiologic roles in adipogenesis, neurogenesis, and vascular development (17-19). Increased expression of ATX has also been reported in many cancers, indicating a stimulatory role

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for this protein in tumorigenesis and metastasis (20, 21). The biological properties of ATX are explained by its lysophospholipase D activity, which produces the potent bioactive phospholipid lysophosphatidic acid (LPA). Moreover, ATX seems to be the predominant enzyme-generating LPA in plasma and serum (22, 23). In addition to LPA production, ATX has also been shown to hydrolyze sphingosylphosphorylcholine (SPC) *in vitro* to yield the potent angiogenic factor sphingosine-1-phosphate (S1P; ref. 24), although the biological significance of this S1P production is uncertain (18).

Expression of the *ATX* gene is strongly stimulated by v-Jun, $\alpha_6\beta_4$ integrin acting through the transcription factor NFAT1, and Hoxa13 (25-27). *ATX* is also regulated in a cell type–dependent manner by growth factors such as fibroblast growth factor, epidermal growth factor, and bone morphogenetic protein 2 (reviewed in ref. 28). Recently, VEGF was identified as a regulator of ATX expression via an autocrine positive feedback loop in ovarian cancer cells (29). Because ATX is the primary source of plasma LPA (18), factors that regulate ATX expression also serve to regulate LPA production. LPA signaling through G protein–coupled receptors (LPA1-LPA5) regulates a wide range of cellular functions, including cell migration, proliferation and survival, as well as ion influx and secretion (reviewed in ref. 21). Experiments in vascular models have shown that LPA is involved in regulation of vascular tone and permeability, and it can also stimulate and attract inflammatory cells (neutrophils and monocytes) as well as platelets (30, 31).

We now show that VEGF via VEGFR2 stimulates ATX expression and consequently LPA production as well as LPA1 signaling in human umbilical vein endothelial cells (HUVEC). Knockdown of *ATX* gene expression in these cells results in reduced expression of the predominant G protein–coupled receptors for LPA and S1P as well as VEGFR2 and Akt2. ATX knockdown also abolishes cell migration to lysophosphatidylcholine (LPC), LPA, recombinant ATX, and VEGF and reduces migration to SPC and S1P. Thus, our data indicate an important role for ATX in endothelial cell migration involving expression of receptors and signaling proteins required for responses to VEGF and lysophospholipids.

Materials and Methods

Cell Cultures

HUVECs and human umbilical arterial endothelial cells (HUAEC) were obtained from Lonza and cultured in endothelial basal medium (EBM) supplemented with EGM Bullet kit, bovine brain extract with heparin, human epidermal growth factor, hydrocortisone, GA-1000 (gentamicin, amphotericin B), and 2% fetal bovine serum according to the instructions provided by the manufacturer. Medium was replaced every other day, and passages 3 to 7 were used for experiments.

Reagents

All chemicals were reagent grade and were obtained from Sigma-Aldrich unless otherwise noted. Oleoyl-L-α-LPA sodium salt was prepared in distilled, deionized water, and 1-oleoyl-sn-glycero-3 phosphocholine (LPC) was prepared in ethanol. SPC and S1P stocks were prepared in methanol and stored at −20°C. To make desired concentrations, stock solutions were combined with DMEM supplemented with fatty acid–free bovine serum albumin (BSA). Human collagen type IV and recombinant VEGFA-165 were from BD Biosciences. Stock solutions of VEGF were prepared in water and stored at −20°C before diluting in DMEM. ATX was expressed in and isolated from High Five insect cells (Invitrogen) or from COS-1 cells as previously described (24). Mouse monoclonal agonistic antibodies for VEGFR1 [hVEGFR1 NH_2-terminal fragment (N25-250)] and VEGFR2 [hVEGFR2 NH_2-terminal fragment (N30-200)] were purchased from Angio-Proteomie, reconstituted according to the protocol provided by the manufacturer in sterile PBS, and stored at −80°C before usage. Recombinant Orf virus VEGF-E was purchased from Cell Sciences and reconstituted according to the protocol provided by the manufacturer in sterile PBS containing 0.1% BSA. Recombinant human placental growth factor (PlGF) was purchased from R&D Systems and reconstituted in sterile PBS containing 0.1% BSA.

Medium Collection for ATX Detection

For each experiment, medium containing transfection reagents was removed, cells were briefly rinsed, then fresh EBM without serum was added, and incubation was continued for an additional 12 to 24 h. Supernatants were collected and concentrated ~100-fold using an Amicon Ultra-4 centrifugal filter device with a 10,000 molecular weight cutoff (Millipore). Western blot analysis of this concentrated supernatant was used to assess ATX secretion.

Western Blot Analysis

Conditioned medium was collected, partially purified, and concentrated as described above. Samples were prepared for immunoblot analysis in lithium dodecyl sulfate sample buffer (Invitrogen) containing β-mercaptoethanol, heated at 95°C for 5 min, and stored at −20°C. Before gel loading, samples (30-35 μg total protein) were heated to 70°C. Electrophoresis was carried out using 4% to 12% NuPage gels and NuPage MOPS running buffer and transferred on polyvinylidene difluoride membranes (0.45 μm pores) in Transfer Buffer (Invitrogen). The membrane was blocked at 4°C overnight in water containing 75 mg/mL glycine, 0.1% Tween 20, and 50 mg/mL nonfat dry milk. ATX was detected using an antipeptide antibody generated in rabbits as previously described (29).

ATX Gene Silencing in HUVEC

Knockdown of the *ATX* gene used antisense morpholino oligomer constructs (GeneTools) with a mismatched oligomer control as described previously in detail (29). Conditions were optimized for HUVEC according to the protocol provided by the manufacturer. Confluent HUVECs from the same passage were treated with 1 μmol/L ATX-specific (ATXMO) or with 1 μmol/L mismatched

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(MisMO) morpholino oligomers. Treatment with carrier alone (Endoporter, GeneTools) served as mock-transfected control. HUVECs at passages 3 to 6 were used for experiments. Transfection was done under standard culture conditions for 16 to 24 h. Gene silencing was measured by comparing secreted protein in conditioned medium from ATXMO-treated cells with that from MisMO-treated controls using Western blot analysis.

LPA1 Gene Silencing in HUVEC

Small interfering RNA (siRNA) targets for LPA1 were identified using siRNA Target Finder algorithm (Applied Biosystems) and constructed with the Silencer siRNA Construction kit using directions supplied by the manufacturer (Applied Biosystems). The target sequence CCGCCGCTTCCATTTTCCT in exon 4 of the human *LPA1* gene (NM_057159) was chosen for use in this study. A scrambled version of the above sequence TCTCCGAACGTGTTCACGT was generated for a specificity control. HUVECs were cultured in T25 flasks until confluent, and siRNA transfection was carried in reduced serum medium Opti-MEM I (Invitrogen) using Lipofectamine 2000 (Invitrogen) and LPA1 siRNA at 2 nmol/L for 6 h. The conditions for transfection were established according to protocol provided by the manufacturer (Invitrogen). After a 6-h incubation with the siRNA complex, fresh culture medium was added and the cells were incubated for another 12 h. To verify the knockdown, RNA was isolated as described below and LPA1 expression was measured by quantitative reverse transcription-PCR (qRT-PCR).

RNA Quantification

Total RNA was isolated using Trizol per the manufacturer's instructions (Invitrogen) and stored at −80°C for downstream applications. RNA for qRT-PCR was generated by a linear amplification of 1 μg total RNA using the MessageAMP II aRNA kit following the procedure supplied by the manufacturer (Applied Biosystems). The T7 RNA polymerase transcription reaction was carried out for 14 h, and RNA yields ranged from 40 to 90 μg aRNA. Reverse transcription of 5 μg aRNA samples was carried out using SuperScript III and random hexamers supplied by the manufacturer (Invitrogen). RNA levels were measured using qRT-PCR (29). The PCR primer sequences for ATX (eNPP2), hypoxanthine phosphoribosyltransferase 1 (HPRT1), VEGF, VEGFR1, VEGFR2, and LPA receptors have been previously reported (29). PCR primer sequences for S1P receptors and Akt isoforms are as follows: S1PR1 (NM_001400), AGCTGTGCTCAACTCCGGCAC and GATGAAGGCCCGACGCATCTC; S1PR2 (NM_004230), TGCGTGGTCCGCTCAAGCCAC and ACGATGGTGACCGTCTTGAGC; S1PR3 (NM_005226), TGGTTCATCGTGTTGGCTGTG and CTGACCAGGCAGTTGCAGACC; S1PR4 (NM_003775), TCTGGGCCTCAGTAGGGCTCC and ATGTTGCCAGGGCATGCATCC; S1PR5 (NM_030760), TGATGGGAGCTTCAGCGGCTC and TGTGGAGCCGCTGGTGTCCAG; Akt1 (NM_001014432), TCGTGTGGCAGCACGTGTACG and TCAGTCTCCGACGTGACCTGG; Akt2 (NM_001626), TCAACTGGCAGGACGTGGTCC and ACCTTGTGTCGACCTCGGACG; and Akt3 (NM_005465), AGGGATCACAGATGCAGCCA and GTCTACTGCTCGGCCATAGTC.

Migration Assays

Migration was done in 48-well modified Boyden chambers (Neuroprobe) as described before in detail with the following modifications (32). Upper and lower chambers were separated by 8-μm pore size polycarbonate filters (Nucleopore) that had been precoated with human collagen type IV at 50 μg/mL in 0.1 mol/L acetic acid. All endothelial cells were suspended at 10^6/mL in EBM containing 1 mg/mL of fatty acid–free BSA and placed in the upper chamber. The chambers were incubated for 3 to 4 h under standard culture conditions. The membranes were fixed and stained as described previously (33). Assays were done in duplicate and quantified by counting three randomly chosen fields under light microscopy. All figures are representative of two or more independent experiments.

Statistical Analysis

Cell counts from motility assays and C_t results (standardized against HPRT1) from qRT-PCR were compared using Prism 3 software (GraphPad Software, Inc.) using one-way ANOVA with either Tukey's or Dunnett's post test or using two-tailed unpaired *t* tests.

Results

Relative ATX Expression in Human Umbilical Venous and Aortic Endothelial Cells

Because vascular regulation of ATX expression is poorly understood, we first analyzed the relative levels of ATX mRNA and protein in three functionally distinct primary endothelial cells: HUVECs, HUAECs, and human dermal microvascular endothelial cells (MVEC). Measurement of steady-state mRNA levels indicated that ATX is expressed in all tested vascular cell types at much lower levels than in ovarian cancer cells (SKOV3) or melanoma cells (MDA-MB-435), which have 20- and 80-fold higher expression, respectively (29). Of the tested endothelial cell types, HUVECs express the highest levels of ATX mRNA, whereas ATX mRNA expression was slightly lower in MVEC and HUAEC (Table 1).

We also analyzed the mRNA expression of VEGF and its receptors for all three endothelial cell types, as well as expression of LPA and S1P receptors. VEGFR1 and VEGFR2 are expressed in all three cell types, with the highest mRNA levels in MVEC (Table 1). Interestingly, only HUVEC expressed LPA receptors (Table 1). In addition, neither HUAEC nor MVEC migrated to the ATX product LPA or to its substrate LPC; however, both migrated to recombinant ATX and to S1P, another product of ATX enzymatic activity (Fig. 1A and B). These data

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Table 1. Cycle threshold (C_t) values from vascular cells used in this study

Gene	HUVEC	HUAEC	MVEC
ATX	32.9 ± 0.24	35.3 ± 0.14	35.9 ± 0.15
VEGF	27.5 ± 0.19	34.1 ± 0.12	26.3 ± 0.43
VEGFR1	33.5 ± 0.23	28.2 ± 0.19	23.5 ± 0.40
VEGFR2	30.1 ± 0.21	28.1 ± 0.06	21.8 ± 0.50
S1P1	19.6 ± 0.08	20.8 ± 0.41	22.2 ± 0.26
S1P2	25.0 ± 0.10	27.1 ± 0.15	21.7 ± 0.13
S1P3	36.8 ± 0.52	33.3 ± 0.65	34.1 ± 0.41
LPA1	25.3 ± 0.01	>38	>38
LPA2	25.3 ± 0.11	>38	>38
LPA3	31.3 ± 0.24	>38	>38
HPRT1	21.0 ± 0.06	22.1 ± 0.11	20.7 ± 0.05

NOTE: Average C_t ± SD; C_t > 38 is beyond the instrument range. HUVEC, HUAEC, and MVEC were cultured under standard conditions for 20 h, and total RNA was assayed for ATX, VEGF, VEGFR1, VEGFR2, S1P1, S1P2, S1P3, LPA1, LPA2, and LPA3 expression using qRT-PCR. HPRT1 served as an internal control in qRT-PCR calculations.

indicate that the available HUAEC and MVEC lack functional LPA signaling. In contrast, the relative expression levels for ATX, LPA1, LPA2, and LPA3 in HUVEC are consistent with their expression in breast endothelium *in vivo* as determined by serial analysis of gene expression[1] (14, 17, 10, and 3/200,000 tags, respectively; ref. 34). Therefore, HUVECs were chosen to analyze regulation of the ATX/LPA axis in endothelial cells.

HUVEC Chemotaxis to Substrates and Products of ATX

ATX was discovered as a potent stimulator of cell migration, and its expression was linked to motility-dependent processes such as cancer invasion and metastasis (20). We examined the migration of endothelial cells to ATX recombinant protein as well as to the products and substrates of its enzymatic activity. Migration of HUVECs was measured in 2.5- to 4.5-hour Boyden chamber assays, and stimulatory effects are shown relative to spontaneous

[1] http://cgap.nci.nih.gov/SAGE/

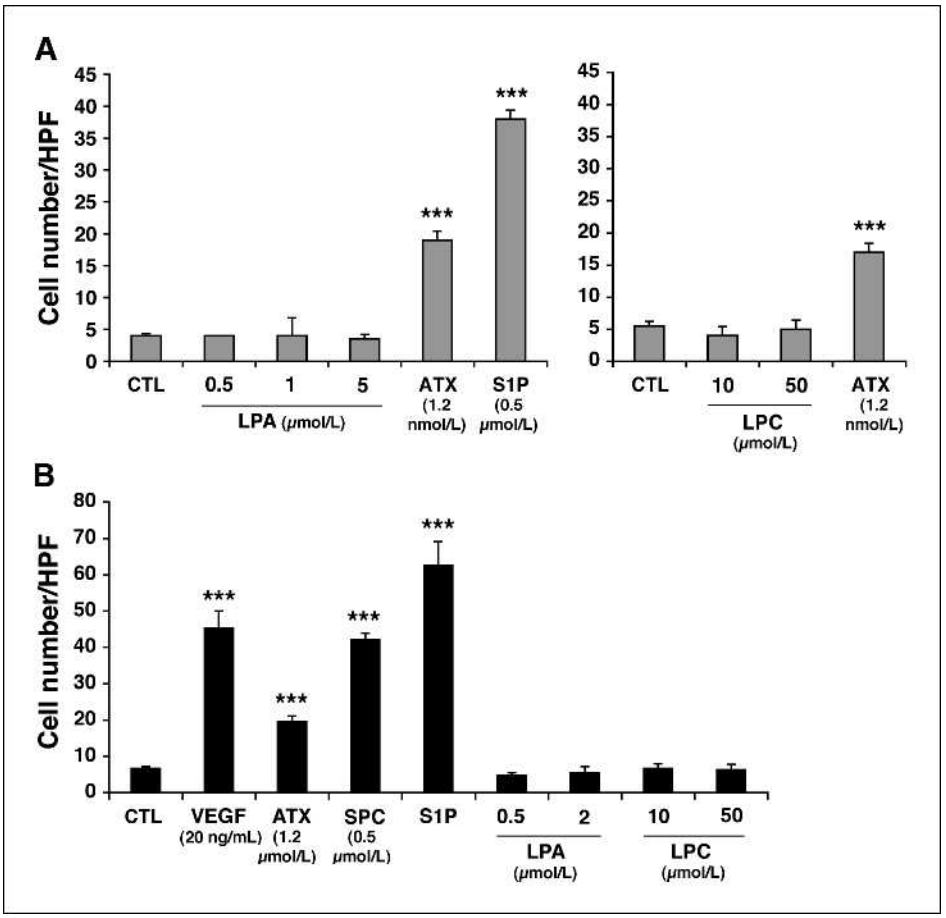

FIGURE 1. A, chemotaxis of HUAEC after 4.5-h exposure to chemoattractants (LPA, ATX, S1P, and LPC) was measured in a modified Boyden chamber assay using collagen type IV–coated filters. Triplicate migration wells were quantified under light microscopy by counting five randomly chosen high-power fields (HPF) and scored as the average number of cells per HPF. The data represent at least two independent experiments. B, chemotaxis of MVEC after 4.5-h exposure to chemoattractants VEGF, ATX, SPC, S1P, LPA, and LPC was measured in a modified Boyden chamber assay using collagen type IV–coated filters. Triplicate migration wells were quantified under light microscopy by counting five randomly chosen HPF and scored as the average number of cells per HPF ± SD. The data represent at least two independent experiments. The results, analyzed by one-way ANOVA with Dunnett's post test comparing all values to that of control (CTL), are shown as ***, $P < 0.001$.

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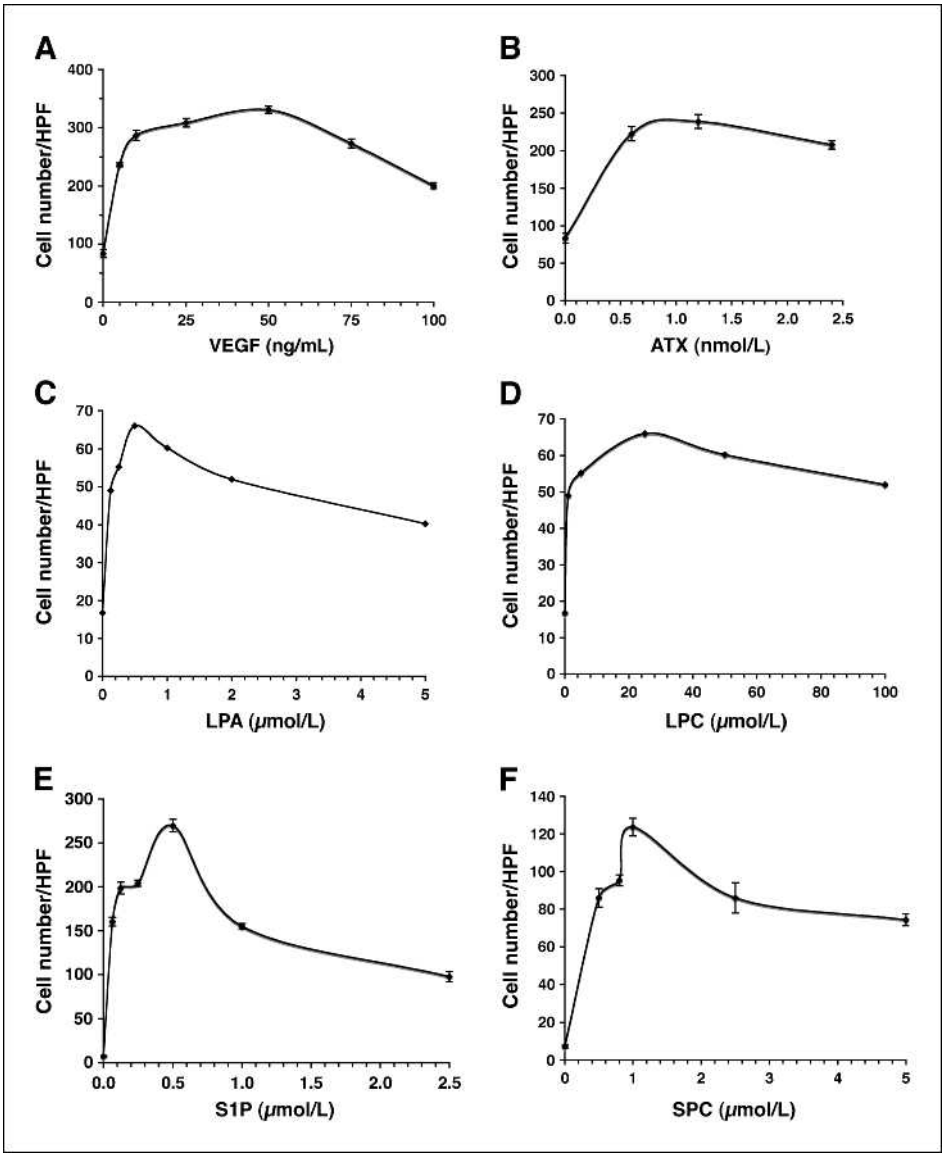

FIGURE 2. Endothelial cell (HUVEC) migration to VEGF, ATX, and ATX products and substrates. Chemotaxis of HUVEC after 4.5-h (except where indicated) exposure to chemoattractants was measured in a modified Boyden chamber assay using collagen type IV–coated filters. Triplicate migration wells were quantified under light microscopy by counting five randomly chosen HPF and scored as the average number of cells per HPF. The data represent at least two independent experiments. A, dose response to recombinant VEGF. B, dose response to recombinant purified ATX. C, dose response to LPC. D, dose response to LPA was assayed after 2.5-h migration. E, dose response to SPC was assayed after 2.5 h. F, dose response to S1P was assayed at 2.5 h. Values are shown as average ± SEM. Responses at all shown concentrations of chemoattractant were statistically greater than background levels.

(background) migration that occurred in the absence of chemoattractant (Fig. 2).

Migration induced by VEGF was maximal at 10 to 50 ng/mL (Fig. 2A). HUVEC also responded well to recombinant purified ATX protein: 0.6 nmol/L resulted in a 2.5-fold stimulation (Fig. 2B). In a previously published report, recombinant ATX protein acted as a weak chemoattractant for endothelial cells (33). However, using collagen type IV, instead of collagen type I or gelatin, to coat the membranes resulted in a significant enhancement of the HUVEC migratory response to recombinant ATX protein at subnanomolar concentrations (Fig. 2B).

LPA also stimulated migration on collagen type IV–coated filters after 2.5 hours and reached 3-fold above background at 0.5 μmol/L LPA (Fig. 2C). A longer incubation time (4.5 hours) resulted in a higher background and a lower net migration, with maximal stimulation 2-fold greater than control (data not shown). Using the same protocol, the ATX substrate LPC maximally stimulated endothelial cell migration 3-fold above background at 20 to 50 μmol/L (Fig. 2D). S1P is a potent stimulator of migration for endothelial cells derived from small and large arteries and veins over a broad range of concentrations (35, 36). S1P is also the enzymatic product of ATX hydrolysis of SPC (24). In our assay system, S1P stimulated HUVEC migration 15-fold at 0.1 μmol/L and reached a maximum at 0.5 μmol/L concentrations (Fig. 2E). SPC stimulated migration and reached a maximum of 12-fold above control at 0.8 to 1 μmol/L (Fig. 2F). It should be noted that migration responses to S1P and SPC were measured with an incubation time of 2.5 hours due to difficulty counting the high number of migratory cells in longer assays. These assays were done

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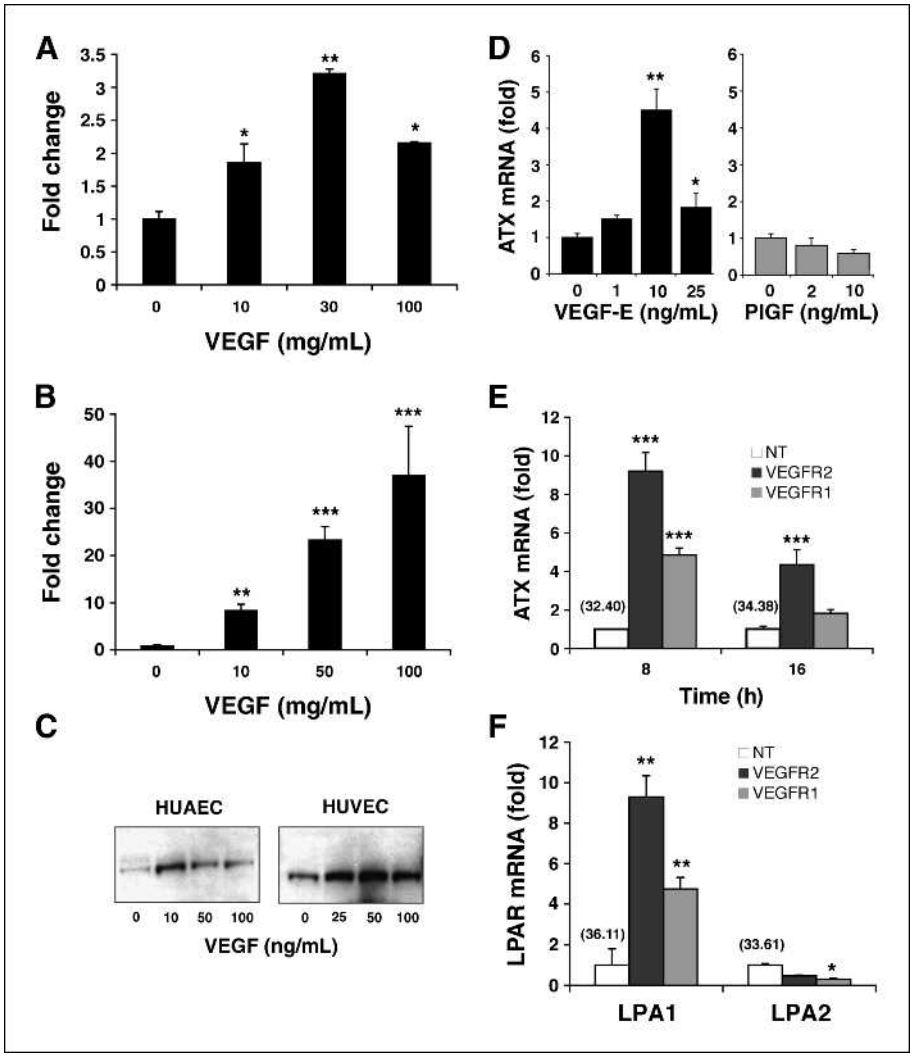

FIGURE 3. VEGF stimulates ATX production in endothelial cells via VEGFR2. ATX mRNA levels were quantified by qRT-PCR in HUVEC treated with the indicated concentrations of recombinant VEGF for 5 h (A) or 16 h (B). C, conditioned medium was collected from HUVEC and HUAEC after treatment with VEGF for 16 h, and ATX protein levels were assessed by Western blot analysis. D, HUVECs were treated with VEGF-E or PlGF at indicated concentration for 8 h, and ATX mRNA levels were measured using qRT-PCR. E, HUVECs were treated with agonistic VEGFR2 or VEGFR1 antibodies in separate reactions for 8 and 16 h. ATX mRNA levels were measured using qRT-PCR. F, HUVECs were exposed to agonistic VEGFR2 or VEGFR1 antibodies for 16 h, and LPA1 and LPA2 mRNA levels were measured by qRT-PCR. Raw C_t values (shown in E and F as numbers in parentheses above data for untreated cells) were adjusted to HPRT1 internal controls and then expressed as fold difference ± SEM. The results of statistical analysis of the HPRT1-adjusted data (via one-way ANOVA with Tukey's post test) are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus untreated control.

to establish the most effective time and concentration of chemoattractants for HUVEC migration. It should also be noted that recombinant ATX and VEGF as well as LPC and LPA produce similar migratory responses that were about 2.5- to 3-fold above control.

VEGF Increases ATX mRNA and Secreted Protein Levels in Endothelial Cells

We have previously shown that VEGF stimulates ATX production in ovarian cancer cells that express VEGF receptors as a part of a pathologic positive feedback loop (29). Based on these findings, we hypothesized that VEGF might also regulate ATX expression in endothelial cells. Secreted ATX protein was measured after endothelial cells were incubated in supplement-free medium for 16 to 24 hours. Cell supernatants were then collected and concentrated. Although Nam et al. (33) had detected ATX protein in concentrated endothelial cell supernatants when cells were grown in the presence of growth factors, our data represent baseline ATX protein expression when cells were grown to confluence under standard culture conditions and then incubated in supplement-free EBM for 16 hours. Western blot analysis revealed that secreted ATX protein could be detected in conditioned medium from each endothelial cell line incubated without growth supplements. Secreted ATX protein parallels the differences seen in mRNA expression (Table 1).

Endothelial cells have been extensively studied in terms of their VEGF responsiveness for both angiogenesis and vascular homeostasis. Previously, Nam et al. (33) reported that treatment with fibroblast growth factor-2 but not VEGF stimulated ATX expression in HUVEC; however, VEGF treatment was done only for 1 hour, and the VEGF concentration was not defined. To more closely examine VEGF regulation of ATX production, HUVEC and HUAEC were treated with recombinant VEGF-165 at various concentrations and times. ATX mRNA levels were measured by qRT-PCR and secreted protein levels by Western blot.

A dose-dependent increase in ATX mRNA was observed in VEGF-treated HUVEC as early as 5 hours, with an

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optimal VEGF concentration of 30 ng/mL (Fig. 3A). However, the greatest stimulation of ATX mRNA was observed after 16 to 18 hours of treatment and was maximal at 100 ng/mL VEGF (Fig. 3B). This increase in ATX mRNA was reflected in higher amounts of secreted ATX protein in the conditioned medium from stimulated cells (Fig. 3C). Treatment of HUAEC with VEGF similarly resulted in a dose-dependent stimulation of ATX protein levels detectable in concentrated cell supernatants after 16 hours, with maximal induction at 10 ng/mL VEGF, 5-fold lower than the effective concentration for HUVEC (Fig. 3C). This effect may be a consequence of higher VEGF receptor expression in HUAEC (see Table 1). Nonetheless, these findings indicate that VEGF stimulates ATX expression and secretion in both endothelial cell types.

Induction of ATX Expression by VEGF Is Mediated by VEGFR2

VEGF-E, PlGF, and agonistic antibodies for VEGFR1 or VEGFR2 were used to assess the relative contribution of each VEGF receptor to the regulation of ATX synthesis in endothelial cells. Treatment of HUVEC for 8 hours with VEGF-E, which stimulates VEGFR2 signaling selectively (37), resulted in increased expression of ATX mRNA (Fig. 3D, left). In contrast, treatment of HUVEC with PlGF, which stimulates VEGFR1 signaling (38), did not affect ATX expression in our assays (Fig. 3D, right). Interestingly, activation of VEGFR2 by its agonistic antibody resulted in an ~9-fold increase in ATX mRNA levels after an 8-hour exposure, whereas stimulation by a VEGFR1 agonist resulted in a 4-fold increase in ATX mRNA (Fig. 3E). Although maximal ATX upregulation by recombinant VEGF protein was observed after 16 hours (data not shown), 16-hour treatment with the VEGFR2 agonist increased ATX mRNA levels ~4-fold, and VEGFR1 agonistic antibody produced no significant change in ATX expression at this time point (Fig. 3E). Taken together, these data indicate that VEGFR2 signaling predominates in the VEGF-stimulated expression of ATX in endothelial cells.

Because ATX is thought to exert its major physiologic effect through the production of LPA, we also examined the effect of VEGF signaling on LPA receptor expression in HUVEC. Stimulation with VEGFR2 agonistic antibody for 16 hours resulted in a 16-fold increase in LPA1 expression, whereas similar treatment with VEGFR1 agonist increased LPA1 expression 9-fold (Fig. 3F). Neither antibody had any effect on LPA2 expression. Therefore, VEGF not only increases ATX production in endothelial cells but also stimulates the response to the ATX product LPA by increasing LPA1 receptor expression.

ATX Knockdown in HUVECs Using Morpholino Oligomers

We next examined the effect of ATX knockdown in HUVEC. The method of transiently reducing ATX expression using morpholino oligomer treatment was previously

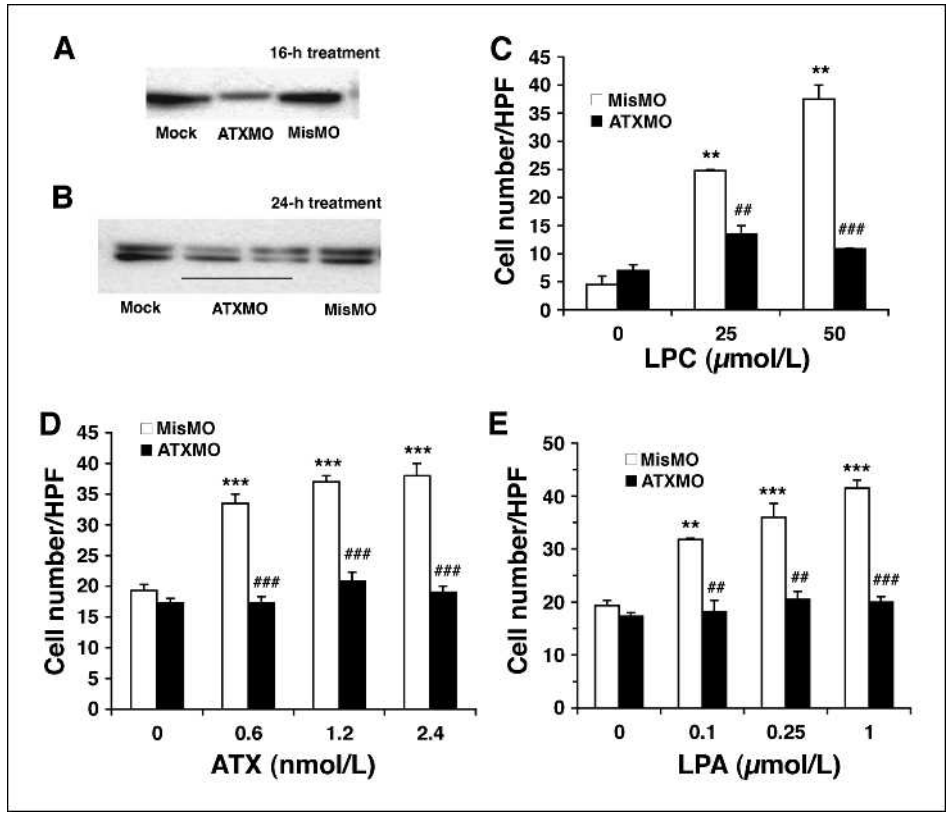

FIGURE 4. ATX knockdown in HUVEC limits migration to LPC, LPA, and recombinant ATX protein. A, Western blot analysis to measure ATX protein levels in conditioned medium from HUVEC after 16-h treatment with 1 μmol/L ATXMO followed by 16-h incubation after removal of oligomers. MisMO and mock transfectants were used as specificity controls and incubated under identical conditions. B, ATX protein levels after 24-h treatment with ATXMO (1 μmol/L) and 24-h incubation following removal of morpholino oligomers. These same cells were assessed for their chemotactic responses to optimal concentrations of LPC (C), recombinant ATX protein (D), and LPA (E). Results are shown as average ± SEM. Statistical analysis for C to E used one-way ANOVA with Tukey's post test. Each treatment group was first compared with its own background motility (**, $P < 0.01$; ***, $P < 0.001$). Then, for each concentration of chemoattractant, ATXMO treatment was compared with MisMO (##, $P < 0.01$; ###, $P < 0.001$).

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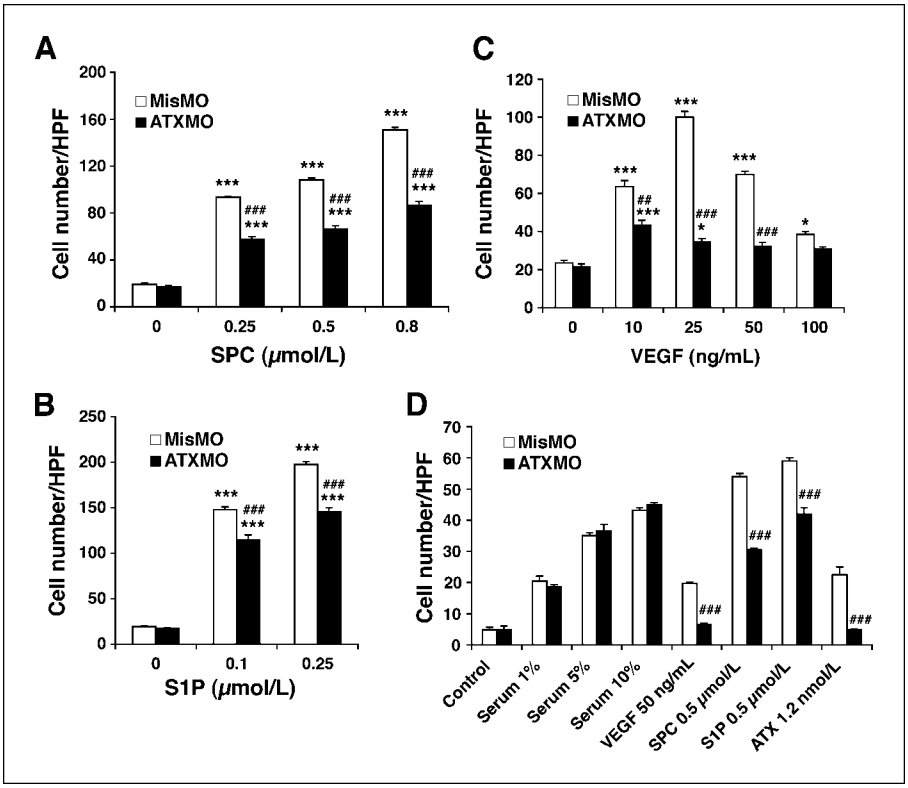

FIGURE 5. Migration to SPC, S1P, VEGF, and serum in ATX knockdown HUVEC. HUVECs were treated with 1 μmol/L ATXMO or MisMO for 24 h followed by a 24-h incubation without oligomers. These cells were assessed for their motility responses in 4.5-h Boyden chamber assays in response to SPC (A), S1P (B), or recombinant VEGF-165 (C). D, migration to serum was not affected by ATX knockdown in a 2.5-h Boyden chamber assay. Single concentrations of SPC, S1P, VEGF, and ATX were used as positive controls to confirm reduced motility of ATX knockdown cells. All results are shown as average ± SEM. Statistical analysis used one-way ANOVA with Tukey's post test. First, each treatment group was compared with its own background motility (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Then, for each concentration of chemoattractant, ATXMO treatment was compared with MisMO (##, $P < 0.01$; ###, $P < 0.001$).

validated in ovarian cancer cells (29). HUVECs were treated with ATXMO under standard culture conditions in duplicate. MisMO as well as mock-treated cells were used as specificity controls. The most effective time of treatment and concentration of oligomers for HUVEC were established empirically. This treatment was followed by oligomer removal and incubation of treated HUVEC for 12 hours in EBM without supplements. To validate the knockdown, concentrated cell supernatants were examined for the presence of ATX by Western blot analysis.

Treatment for 16 hours using 1 μmol/L ATXMO was sufficient to achieve a 50% to 60% reduction in ATX protein expression compared with MisMO without affecting cell viability (Fig. 4A).

A longer (24-hour) exposure to the morpholinos at the same concentration, followed by identical 12-hour incubation in basal medium, did not result in a more complete knockdown than the 16-hour exposure (Fig. 4B). Therefore, the 16-hour treatment was used in subsequent experiments. The time frame in which ATX expression is successfully reduced in endothelial cells was established to be 12 to 16 hours after removal of ATXMO, so all biological experiments in HUVEC with morpholino knockdown of ATX were done within this time frame.

Reduction in ATX Expression Limits Endothelial Cell Migration

Reduction of ATX expression in ovarian cancer cells resulted in significant inhibition of cell migration to ATX, lysoglycerophospholipids, and VEGF (29). Using the optimized assay conditions from Fig. 2, LPC-stimulated migration in ATXMO-treated HUVEC was reduced to background levels (Fig. 4C). This result was expected because LPC stimulation of migration is presumed to be dependent on the enzymatic activity of ATX (39). However, exogenously added LPA did not restore migration in ATX knockdown HUVEC (Fig. 4E). Instead, LPA-stimulated migration in ATXMO-treated HUVEC was not significantly different than that of control cells (Fig. 4E). Similarly, migration to exogenously added recombinant ATX protein was reduced to background levels (Fig. 4D). Background migration in ATXMO-treated HUVEC was not significantly different than that of MisMO-treated cells in any of the experiments shown in Fig. 4 or 5. These experiments showed that neither recombinant ATX nor its exogenously added product (LPA) could restore migration in ATX-deficient endothelial cells. This was true for both 2.5-hour and 4-hour assay times.

Because SPC is another ATX substrate, we examined whether ATX knockdown would affect migration to SPC. Interestingly, response to SPC of ATXMO-treated cells was also significantly reduced by 40% to 50% (Fig. 5A). Furthermore, when another ATX product, S1P, was used as chemoattractant, only 70% to 80% of migration was restored in ATX knockdown cells compared with the MisMO control. The observed 20% to 30% inhibition was statistically significant, indicating that

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exogenously added S1P did not completely restore migration in ATX-deficient HUVEC (Fig. 5B).

The effect of ATX reduction on endothelial cell migration was further shown when purified VEGF protein was used as an attractant (Fig. 5C). Recombinant VEGF stimulated a very weak response in ATX knockdown HUVEC. Migration was significantly decreased at 10 to 25 ng/mL VEGF and was reduced to levels that were not significantly above background at higher concentrations of VEGF (Fig. 5C).

The effects of ATX knockdown on HUVEC motility responses to recombinant ATX, its phospholipid substrates and products, and the growth factor VEGF indicated an important role for ATX in endothelial cell migration. The inhibition of endothelial cell migration was not due to a cytotoxic effect of this knockdown because ATX-MO-treated cells had background motility identical to that of MisMO-treated cells, and they exhibited a normal migratory response to serum (Fig. 5D). Therefore, ATX knockdown limits migratory responses only for specific chemoattractants.

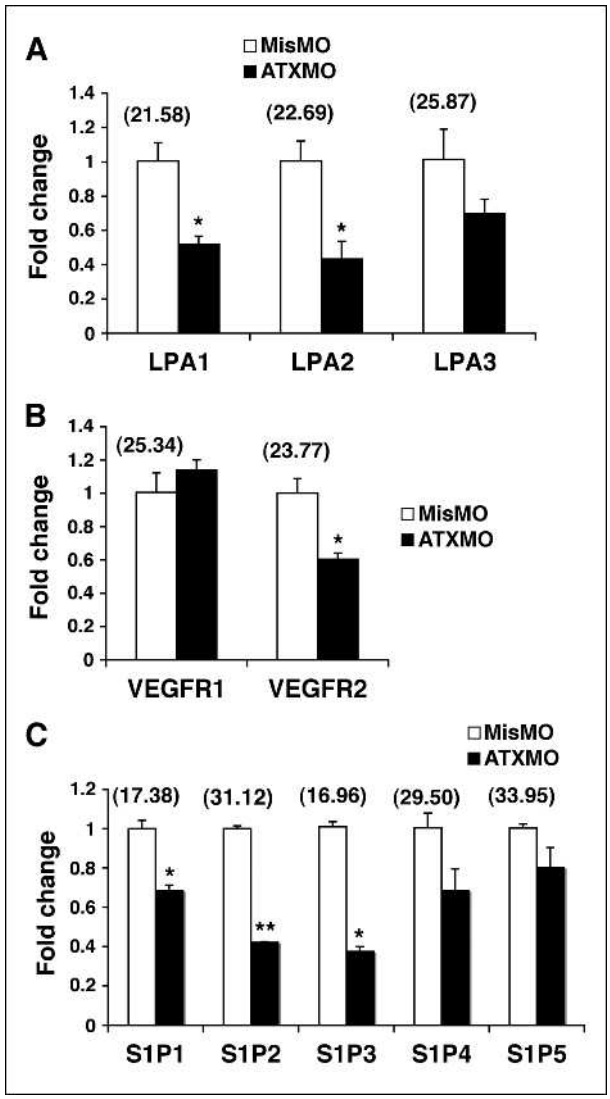

FIGURE 6. Effects of ATX knockdown on LPA, S1P, and VEGF receptor expression in HUVEC. Effects of ATX knockdown on mRNA expression levels were measured by qRT-PCR for LPA receptors (A), VEGFR1 and VEGFR2 (B), and S1P receptors (C). Numbers above control bars indicate raw C_t number for untreated controls (MisMO-treated cells). Statistical analysis of data was first standardized to HPRT1 controls, and then, comparisons of ATXMO- to MisMO-treated cells used unpaired two-tailed *t* tests. *, $P < 0.05$; **, $P < 0.01$.

ATX Knockdown Limits LPA, S1P, and VEGF Receptor Expression in HUVEC

This selective dependence of HUVEC motility on ATX could be explained if ATX is required to maintain expression of receptors or downstream signaling molecules required for specific motility responses. Therefore, expression of LPA, S1P, and VEGF receptors was measured using qRT-PCR in ATX knockdown HUVEC. Significantly reduced expression of LPA1 and LPA2 occurred in ATX-MO-treated cells, but any difference in LPA3 expression was not statistically significant (Fig. 6A). LPA4 expression was below detection limits (data not shown). These data imply that ATX promotes the expression of LPA1 and LPA2 receptors, which provides a possible explanation for the decreased migration to LPC, LPA, and recombinant ATX after ATX knockdown.

ATXMO treatment also resulted in statistically significant reduction in VEGFR2 mRNA levels but had no significant effect on VEGFR1 mRNA (Fig. 6B). Similarly, the reduced migration to S1P could be explained by decreased expression of S1P receptors (Fig. 6C). Expression of S1P1 was reduced to ~70% of its level in MisMO-treated cells, larger decreases were observed for S1P2 (50% of control; $P < 0.01$) and S1P3 (60% of control; $P < 0.05$), whereas changes in S1P4 and S1P5 were not statistically significant (Fig. 6C). Taken together, these results show that ATX expression in endothelial cells is limiting for the expression of VEGFR2, two G protein–coupled LPA receptors, and three S1P receptors.

ATX Signaling via LPA1 Maintains Akt Expression in HUVECs

Endothelial cell receptors for VEGF and S1P stimulate cellular motility partly via the phosphoinositide 3-kinase (PI3K)/Akt pathway (40, 41). In ATXMO-treated HUVEC, the steady-state mRNA expression of Akt2 was decreased ~5-fold and Akt3 expression was reduced ~3-fold compared with their respective MisMO-treated controls, whereas Akt1 was not significantly changed (Fig. 7A). Because LPA is thought to mediate most physiologic effects of ATX, we further examined the effects of LPA on the expression of Akt isoforms. Incubation of endothelial cells with LPA for 8 hours resulted in a weak biphasic stimulation of Akt1 and Akt3 mRNA levels that were not statistically significant (Fig. 7B). In contrast, Akt2 mRNA expression increased in a biphasic manner with a maximal 20-fold increase at 0.2 μmol/L LPA (Fig. 7C). Among the three *AKT* genes, *AKT2* is expressed at the lowest basal level in HUVEC

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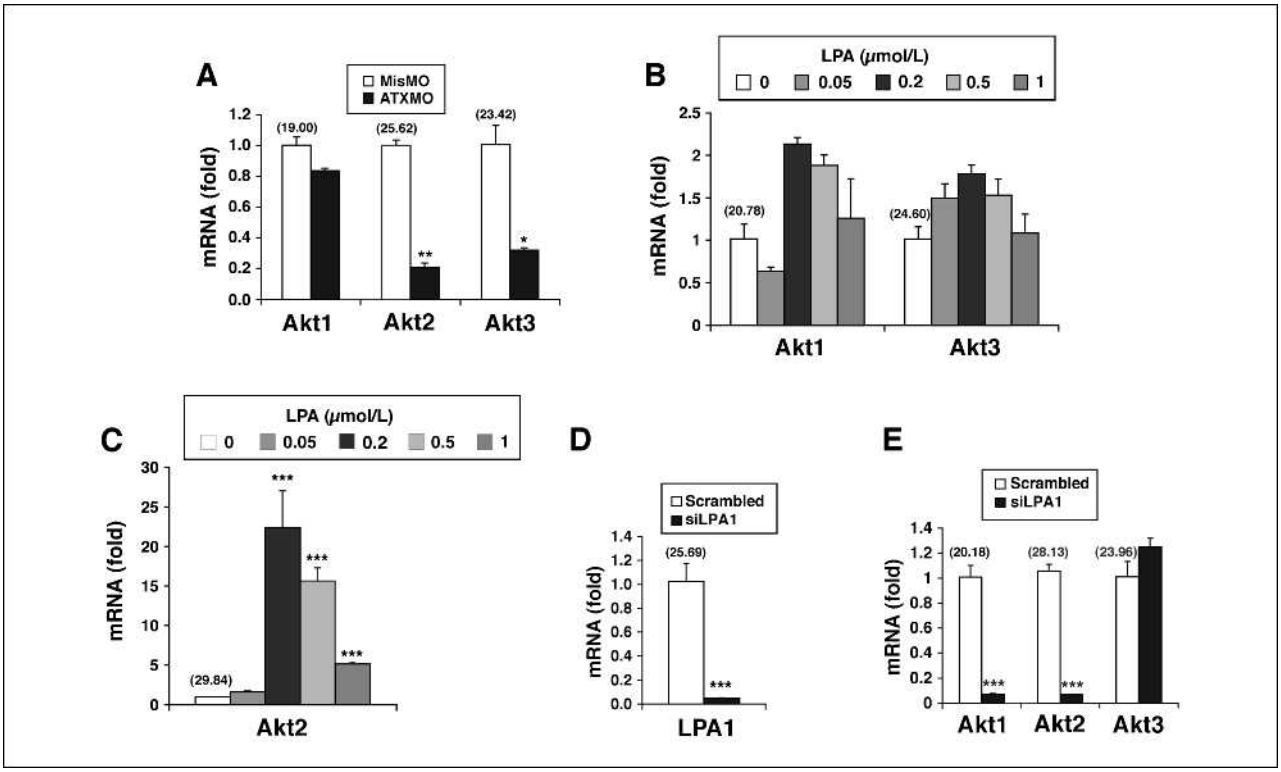

FIGURE 7. Effects of ATX knockdown or siLPA1 treatment on expression of Akt isoforms in HUVEC. A, qRT-PCR was used to measure Akt1, Akt2, and Akt3 mRNA levels in ATXMO-treated HUVEC. Next, HUVECs were treated with indicated concentrations of LPA for 16 h, and then qRT-PCR was done to measure Akt1 and Akt3 expression (B) or Akt2 expression (C). Finally, treatment of HUVEC with LPA1-specific siRNA resulted in >10-fold decrease in LPA1 expression (D) and changes in Akt1, Akt2, and Akt3 mRNA levels (E). Statistical analysis for A, D, and E used unpaired two-tailed *t* tests and for B and C used one-way ANOVA with Dunnett's post test. In all cases, results, compared with control (MisMO in A, untreated in B and C, and scrambled siRNA in D and E), are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 7A), but treatment with LPA strongly stimulated its expression, whereas knockdown of ATX strongly decreased its expression.

Because treatment of HUVEC with VEGFR2 agonistic antibody stimulated expression of LPA1 in HUVEC (see Fig. 2E), we tested whether downregulation of LPA1 expression would affect Akt expression. LPA1 expression was reduced by 80% using LPA1-specific siRNA and qRT-PCR was done to validate the knockdown (Fig. 7D). Expression of the Akt isoforms in LPA1-deprived HUVEC resulted in a 10-fold reduction in Akt2 mRNA levels, a 5-fold reduction in Akt1 mRNA levels, and no effect on Akt3 expression (Fig. 7E). These data indicate that LPA predominantly stimulates expression of Akt2 in endothelial cells via the LPA1 receptor. VEGF stimulation of ATX expression and consequent LPA production and signaling could stimulate endothelial cell migration via this pathway.

Discussion

We found that treatment of either HUVEC or HUAEC with VEGF significantly increases ATX expression. These data paralleled the induction of ATX in VEGF-treated ovarian cancer cell lines (29), suggesting that VEGF may be an important regulator of ATX expression in several cell types. In addition, VEGF stimulates endothelial cell expression of LPA1, a signaling receptor for the ATX product LPA. Morpholino oligomer reduction of ATX expression revealed that migration of HUVEC to LPA/LPC, S1P/SPC, VEGF, and ATX requires sustained ATX expression. This requirement can be explained by the important role that the ATX product LPA plays in maintaining mRNA levels for the G protein–coupled receptors LPA1, LPA2, S1P1, S1P2, and S1P3, VEGFR2, and Akt2 in HUVEC. Our previous findings and current data suggest that ATX expression is necessary for motility responses to VEGF in both HUVEC and ovarian cancer cells (29).

VEGF is a crucial angiogenic factor that regulates multiple biological functions in endothelial cells. In ovarian cancer cells, ATX knockdown using morpholino oligomers resulted in attenuated motility responses to ATX, LPC, LPA, and VEGF (29). ATXMO treatment of HUVEC causes an even stronger reduction in these migratory responses, which are reduced to background or near-background levels. Our data suggest that VEGF, VEGFR2, ATX/LPA, and LPA receptors form a regulatory loop that could augment tumor-related angiogenesis and metastasis. This loop could be initiated by secretion of VEGF into the

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tumor microenvironment by both cancer cells and stromal cells. VEGF induces upregulation of ATX expression, which in turn increases LPA production. The simultaneous induction of LPA receptors allows LPA to stimulate both VEGFR2 and Akt isoform expression (particularly Akt2), thus enhancing cellular responses to VEGF and stimulating endothelial cell migration (summarized in Fig. 8). Unlike ovarian cancer cells, HUVECs also migrate in response to the ATX substrate/product pair SPC and S1P. ATXMO-treated HUVEC displayed a smaller but significant inhibition in their migratory response to S1P and SPC, which may be explained by reductions in mRNA levels for several S1P receptors and for their Akt effectors. Although in this study we have focused on the LPA-dependent mechanism of this regulatory loop, our data do not exclude the possibility of local ATX-derived S1P production or the existence of ATX/S1P-dependent pathways. Taken together, our data suggest that upregulation of ATX expression by VEGF, and possibly by other angiogenic growth factors, could be an important mechanism in pathophysiologic conditions.

Both VEGF and S1P have been shown to stimulate endothelial cell migration via activation of Akt (40, 41). In addition, these two molecules have been shown to act together in primary endothelial cells to stabilize Akt3 mRNA and to increase its expression (42). Dimmeler and coworkers (43) showed that overexpression of constitutively active Akt was sufficient to induce endothelial cell migration. Similarly, PI3K inhibitors disrupted LPA-induced migration in vascular endothelial cells (35, 44). Here, we show that both Akt2 and Akt3 mRNA expression are significantly decreased by ATX knockdown in HUVEC, whereas stimulation of HUVEC with the ATX product LPA resulted in a 20-fold increase in Akt2 expression. Interestingly, silencing the *LPA1* gene via siRNA resulted in decreased expression of both Akt1 and Akt2. Thus, ATX, through its product LPA and the LPA1 receptor, seems to play an important regulatory role in controlling the relative expression of the three Akt isoforms. Akt2 was recently found to be selectively recruited to the leading edge of migrating endothelial cells and to be activated at this site (45). Our data suggest that Akt2 mRNA is directly regulated by the VEGF/ATX/LPA/LPA1 pathway, whereas LPA may affect the regulation of Akt1 and Akt3 mRNA expression by acting in conjunction with other factors.

The three Akt/protein kinase B family members are serine/threonine kinases that are regulated by PI3K signaling. Akt has important roles in growth, survival, differentiation, metabolism, migration, and other cellular activities, and the PI3K/PTEN/Akt pathway is frequently dysregulated in cancers (46). *AKT2* is amplified frequently in pancreatic (47), late-stage colon (48), and ovarian (49) carcinomas. Interestingly, Akt2 activation has been correlated to increased motility and invasiveness in gliomas (50) and breast cancer cells (51) and is associated with increased metastatic potential in colon cancers (52). In breast cancer cells *in vitro*, Akt2 was found to stimulate both a migratory response and epithelial-mesenchymal transition in opposition to their inhibition by Akt1 (53). Akt2 overexpression specifically increases lung metastasis of breast carcinoma *in vivo* (54). ATX expression has been correlated with tumor cell motility and metastasis, as well as tumor-related angiogenesis, and the preferential effect of ATX and LPA on expression of Akt2 versus Akt1 mRNA could be an important effector pathway for this function of ATX.

We propose that VEGF and potentially other angiogenic growth factors that induce ATX expression can activate this

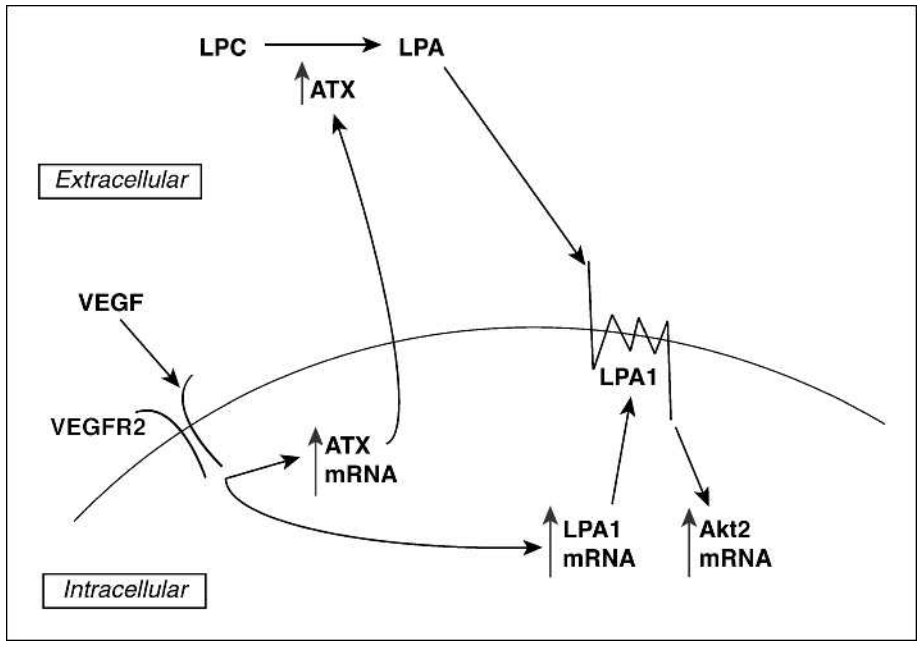

FIGURE 8. Proposed pathway in regulation of ATX expression, LPA production, and signaling by VEGF in HUVEC. Cancer cells and stromal cells secrete VEGF, which via VEGFR2 stimulates expression of ATX and extracellular LPA production, as well as mRNA levels for LPA1 receptor. The elevated extracellular ATX would increase localized levels of LPA and stimulate LPA signaling, particularly via LPA1. Elevated LPA1 signaling, in turn, increases Akt2 mRNA levels. Enhanced expression of Akt2 has been associated with cancer progression, cellular migration, and promotion of epithelial-mesenchymal transition.

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proangiogenic response. Other growth factors that regulate ATX expression include fibroblast growth factor-2, epidermal growth factor, and bone morphogenetic protein 2 (reviewed in ref. 28). Thus, drugs that limit ATX expression or activity could have broader antiangiogenic activities than the currently approved antiangiogenic drugs. Although the ATX product LPA stimulates endothelial cell migration and proliferation *in vitro*, it is not considered to be an angiogenic factor because transgenic mice lacking genes for one or both prominent LPA receptors (*LPA1* and *LPA2*) do not have defective vascular development. However, this lack of vascular phenotype may be explained by the recent demonstration that p2y5/LPA6 plays a significant role in endothelial cell responses to LPA (55). In contrast, transgenic *ATX*-null mice die at embryonic day 10.5 due to impaired vasculogenesis (56). Because ATX has an essential role in vascular and neurologic development, this observation raises the question of whether production of LPA is indeed the only function of ATX. The regulatory functions of ATX suggested by our data could have important implications for tumor angiogenesis and metastases formation, with potential for new strategies in prognostic evaluation or therapeutic intervention. The angiogenesis inhibitors currently in clinical use for cancer all target VEGF or its receptor VEGFR2. Although these drugs extend patient survival, cancers eventually overcome this inhibition, and side effects including hypertension and thrombosis limit their continued use (57). Therefore, the identification of additional genes that are required for angiogenesis could be exploited as targets for the next generation of tumor angiogenesis inhibitors. ATX is one potential target based on its ability to stimulate tumor angiogenesis (33), its critical role in developmental blood vessel formation (56), its importance as the major source for the circulating LPA (56), and correlation of its expression with poor prognosis in several cancers (58-64).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Autotaxin Signaling via Lysophosphatidic Acid Receptors Contributes to Vascular Endothelial Growth Factor–Induced Endothelial Cell Migration

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