Autotaxin Stabilizes Blood Vessels and Is Required for Embryonic Vasculature by Producing Lysophosphatidic Acid*

Received for publication, May 30, 2006, and in revised form, July 5, 2006 Published, JBC Papers in Press, July 6, 2006, DOI 10.1074/jbc.M605142200

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Autotaxin (ATX) is a cancer-associated motogen that has multiple biological activities in vitro through the production of bioactive small lipids, lysophosphatidic acid (LPA). ATX and LPA are abundantly present in circulating blood. However, their roles in circulation remain to be solved. To uncover the physiological role of ATX we analyzed ATX knock-out mice. In ATX-null embryos, early blood vessels appeared to form properly, but they failed to develop into mature vessels. As a result ATX-null mice are lethal around embryonic day 10.5. The phenotype is much more severe than those of LPA receptor knock-out mice reported so far. In cultured allantois explants, neither ATX nor LPA were angiogenic. However, both of them helped to maintain preformed vessels by preventing disassembly of the vessels that was not antagonized by Ki66425, an LPA receptor antagonist. In serum from heterozygous mice both lysophospholipase D activity and LPA level were about half of those from wild-type mice, showing that ATX is responsible for the bulk of LPA production in serum. The present study revealed a previously unassigned role of ATX in stabilizing vessels through novel LPA signaling pathways.

Autotaxin (ATX) is a motogen-like phosphodiesterase originally isolated from conditioned medium of human melanoma cells (1). Enforced expression of ATX in Ras-transformed NIH3T3 cells greatly enhances their invasive, tumorigenic, and metastatic potentials (2). In addition, enhanced expression of ATX has been demonstrated in various malignant tumor tissues (3). Thus, ATX is implicated in tumorigenic and metastatic potentials of cancer cells. ATX is also expressed in various tissues and is present at high concentration in various biological fluids including plasma, serum, and seminal plasma (4), implying specific roles of ATX in circulation.

Recently, ATX was shown to have lysophospholipase D (lysoPLD) activity, which converts lysophosphatidylcholine to a bioactive lysophospholipid, lysophosphatic acid (LPA) (5, 6). ATX also converts sphingosylphosphorylchlone into another bioactive lysophospholipid, sphinosine 1-phosphate (S1P) in vitro (7). Because LPA and S1P are regulators of cell motility and proliferation in various cell systems, they might be the effectors of the motogenic actions of ATX. LPA and S1P have been shown to have diverse roles in many biological processes that are mediated by G protein-coupled receptors (GPCRs) specific to LPA or S1P; there are five GPCRs for LPA (LPA1–5) and five for S1P (S1P1–5) with a number of putative GPCRs (8). Thus, ATX may exert its functions through these receptors. Indeed, ATX stimulates cell motility of tumor cells through one of the LPA receptors, LPA1 (9), and ATX positively or negatively modulates cell motility depending on S1P receptor subtypes (7, 10). To uncover the physiological role of ATX and to identify the endogenous product of ATX, we investigated ATX knock-out mice. In this study we show that ATX produces LPA, but not S1P, in circulating blood and that it contributes to blood vessel stability through novel LPA signaling pathways.

MATERIALS AND METHODS

**ATX Knock-out Mice—** ATX knock-out mice (atx−/−) in the genetic background 129/SvEvBrd were produced by and obtained from Lexicon Genetics (The Woodlands, TX). The ATX gene was targeted with an ATX gene-targeting vector, which was designed to replace the initiation codon and first 45 amino acids encoded by exons 1 and 2 with a lacZ-neo cassette in the vector pKOS (Fig. 1A). The ATX knock-out mice were backcrossed with C57BL/6J mice at least six times before being used. Mice were genotyped by both Southern blotting and PCR of genomic DNA. The PCR primers for detection of wild-type alleles are 5′-cggactctctccgatac-3′ and 5′-tccacattagcggatac-3′, and those for detection of mutant alleles are 5′-gacgcgcttgctct-3′ and 5′-tccacattagcggatac-3′. Flk-1-laxZ knock-in mice (11) were kindly donated by Dr. Janet Rossant (The Hospital for Sick Children, Toronto, Canada). All mice used in this study were bred and maintained at the Animal Care Facility in the Graduate School of Pharmaceutical Sci-

*This research was supported by grants from National Institute of Biomedical Innovation, Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Corporation, the 21st Century Center of Excellence Program, and the Ministry of Education, Science, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2. The abbreviations used are: ATX, autotaxin; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; PECAM, platelet-endothelial cell adhesion molecule; Flk, embryonic day; lysoPLD, lysophospholipase D; RT-PCR, reverse transcription PCR.
ences, the University of Tokyo, under specific pathogen-free conditions in accordance with institutional guidelines.

Recombinant ATX—Two recombinant mouse ATX proteins (wild-type ATX and catalytically inactive T209A mutant ATX) with a His tag at the N terminus were expressed and purified using a baculovirus system and nickel column chromatography (HisTrap HP; GE Healthcare), respectively.

Preparation of ATX-depleted Serum—To establish anti-mouse ATX monoclonal antibody, the recombinant mouse ATX protein (50 μg) was used to immunize rats (WKY/Izm strain) with Freund’s complete adjuvant into the hind footpads. Cells from the enlarged medial iliac and inguinal lymph nodes were fused with mouse myeloma (PAI) cells. The antibody-secreting hybridoma cells were selected by screening with an enzyme-linked immunosorbent assay and immunoprecipitation. One clone (5E5 (rat IgG1)) was found to have activity to immunoprecipitate ATX in mouse serum. 5E5 reacted with mouse, human, and bovine ATX but does not react with rat ATX. The monoclonal antibody was purified from culture supernatant of hybridoma cells and was coupled to Sepharose 4B beads (2 mg/ml) (GE Healthcare). To deplete ATX from mouse serum, mouse serum (1 ml) was incubated with the 5E5-Sepharose 4B (40 μl) for 2 h at 4 °C, and the resulting supernatant was used for measuring lysoPLD activity and LPA production. ATX bound to the 5E5-Sepharose 4B was eluted with 100 mM glycine, pH 2.5. This gave rise to a single 100-kDa band on sodium dodecyl sulfate polyacrylamide gel electrophoresis, showing that 5E5 is specific to ATX.

Immunohistochemistry, Whole-mount Immunostaining, and Whole-mount LacZ Staining—Tissue sections (5 μm) were dehydrated, embedded in paraffin, incubated in 3% (v/v) H2O2 for 20 min, incubated overnight at 4 °C with first antibodies (EPOS anti-a-SMA/HRP; DAKO), stained with diaminobenzidine according to the manufacturer’s protocol, and counterstained with hematoxylin. For whole-mount embryo immunostaining, embryos were fixed in 4% paraformaldehyde, dehydrated, incubated with 5% hydrogen peroxide in methanol, rehydrated through a methanol series to phosphate-buffered saline, incubated in phosphate-buffered saline containing 4% bovine serum albumin and 0.1% Triton X-100, incubated with rat anti-PECAM monoclonal antibody (BD Biosciences), incubated with peroxidase-conjugated goat anti-rat IgG (American Qualex), and stained with diaminobenzidine as a peroxidase substrate. LacZ staining was performed as described (11).
**Autotaxin Knock-out Mice**

![Figure A](image1.png)

**FIGURE 2.** Lysophospholipase D activity in plasma and amniotic fluids. A, lysosphospholipase D activity of plasma and amniotic fluids isolated from atx\(^{+/+}\) and atx\(^{+/−}\) mouse plasma. Plasma samples from four different individual mice were loaded in each lane (10 \(\mu\)g/lane). Activity was determined as described (12–14). B, Western blot of ATX in plasma isolated from atx\(^{+/+}\) and atx\(^{+/−}\) adult mice. Plasma samples from four different individual mice were loaded in each lane (10 \(\mu\)g/lane). Note that lysoPLD activities in amniotic fluids are comparable with those in plasma. C, Western blot analysis of ATX in amniotic fluids isolated from atx\(^{+/+}\) mouse plasma relative to atx\(^{+/−}\) mouse plasma. Results represent the percentage of ATX protein expression in plasma isolated from atx\(^{+/+}\) mouse plasma relative to atx\(^{+/−}\) mouse plasma. Activity was determined as described (12–14). D, S1P level in plasma from atx\(^{+/+}\) and atx\(^{+/−}\) mice. Plasma samples from four different individual mice were loaded in each lane (10 \(\mu\)g/lane). Note that ATX expression was detected in amniotic fluids but not in whole lysates of embryos and yolk sacs. Statistical significance was analyzed using Student’s t-test. Statistically significant differences (p < 0.05) are indicated by asterisks in panels A and B. E, for Western blot analysis the same gels were stained with Coomassie Brilliant Blue and used as loading controls in panels B, D, and E (data not shown).

**Allantois Culture**—Allantoic fluids were isolated from embryos at E8.5 and cultured in Dulbecco’s modified Eagle’s medium containing glutamine and antibiotics (18). To perform the vessel formation, the allantoic explants were cultured for 24 h (37°C; 5% CO\(_2\)) in the presence or absence of the factors tested. In some cases, to examine the effect of factors on stabilization of preformed vessels the allantois were first cultured for 24 h in the presence of amniotic fluids (isolated from E12.5 embryos) to allow formation of stable vascular networks and then cultured for an additional 18 h in the presence or absence of the factors tested. Cells were fixed with 4% paraformaldehyde and then immunostained with anti-PECAM monoclonal antibody.

**Determination of LPA and S1P Concentration, LysoPLD Activity, and Western Blotting**—LPA and S1P concentrations in plasma and serum were determined as described previously (12–14). LysoPLD activity was determined as described using 14:0 lysophosphatidylcholine as substrate (5). Western blotting of ATX was performed as described using ATX-specific monoclonal antibody (15).

**In Situ Hybridization**—Expression of ATX and LPA\(_1\) was detected in paraffin-embedded or frozen sections by in situ hybridization using digoxigenin-labeled RNA probes for ATX and LPA\(_1\), as previously described (16). Specimens were treated with proteinase K at 2 \(\mu\)g/ml in phosphate-buffered saline for 5 min and then fixed in 4% paraformaldehyde/phosphate-buffered saline for 20 min, followed by acetylation. Finally, digoxigenin was immunodetected with 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science).

**Quantitative RT-PCR**—From embryos and yolk sacs at E8.5–E10.5, total RNA was isolated using ISOGEN (Nippongene, Toyama, Japan) and reverse transcribed using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Primers used to detect ATX, LPA receptors, glyceraldehyde-3-phosphate dehydrogenase, and \(β\)-actin were described previously (17).

Quantitative RT-PCR was performed as described (9) using ABI PRISM7000 sequence detection system (Applied Biosystems). Each sample was normalized to the number of \(β\)-actin or glyceraldehyde-3-phosphate dehydrogenase transcripts.
RESULTS

Vascular Defects in ATX-null Embryos—To understand the physiological role of ATX, we investigated atx knock-out mice. The ATX gene was targeted with an ATX gene-targeting vector that was designed to replace the initiation codon and first 45 amino acids encoded by exons 1 and 2 with a lacZ-neo cassette in the vector pKOS (Fig. 1A). The generation of mutant mice was confirmed by Southern blotting (not shown) and polymerase chain reaction (PCR) analyses of tail genomic DNA (Fig. 1B). RT-PCR analysis of embryos carrying the knock-out allele confirmed that ATX mRNA was not produced from the disrupted locus (Fig. 1C). The plasma lysoPLD activity and plasma ATX protein level in atx+/− mice were about half the values found in atx+/+ mice (Fig. 2, A and B), showing that ATX is responsible for most of the lysoPLD activity in plasma. The plasma LPA concentrations in atx+/− mice were about half of those in atx+/+ mice (Fig. 3A). In addition, in ATX-depleted mouse serum, which is prepared by mixing the mouse serum with Sepharose 4B beads coupled with anti-ATX monoclonal antibody (5E5), both lysoPLD activity and LPA production during incubation at 37 °C were hardly detected (Fig. 3, B and C). These results clearly indicate that ATX is a major enzyme that produces LPA in serum. By contrast, the S1P levels in plasma (Fig. 3D) and serum (not shown) from atx+/−/mice were unchanged. Despite the reduced LPA level, heterozygous mice appeared phenotypically normal. Viability and fecundity were similar to those in wild-type mice.

Intercrossing of heterozygous animals produced no homozygous pups, indicating that ATX mutations are recessive embryonic lethal. To determine when homozygous embryos were dying, embryos were isolated at various stages of gestation. Homozygous embryos survived at least to embryonic day 10.5 (E10.5) (Table 1). From E9.5, homozygous embryos were clearly abnormal, and at E11.5 almost all of them were dead in utero. Several defects were evident in the homozygous embryo proper. These included growth retardation, open and kinky neural tubes, pericardial effusion, reduced number of somites, incomplete turning, pallor, and fragility (Fig. 4). The hearts beat until E10.5, but contractions were weak and irregular. In addition, yolk sacs were apparently abnormal in homozygous (atx−/−) embryos. The yolk sac of atx−/− embryos completely lacked large vitelline vessels at E9.5 and E10.5, whereas wild-type (atx+/+) yolk sacs had well developed vitelline vessels (Fig. 5A). Histological analysis also showed a clear difference in morphology between atx+/+ and atx−/− tissues. Individual vessels were clearly visible in atx+/+ tissue, with numerous regularly spaced attachments between the visceral endoderm and mesoderm layers (Fig. 5A). Visceral endoderm and mesoderm were more widely separated in atx−/− tissue, with occasional attachments forming numerous large spaces that were sparsely populated with blood cells (Fig. 5A). Despite the vascular defects, embryonic erythrocytes were present (Fig. 5A, arrows), indicating that blood cells differentiated. To specifically visualize the endothelial vasculature in the developing yolk sac and embryo, we utilized flk-1 heterozygous mice, in which expression of lacZ is regulated by the promoter of the flk-1 gene and is restricted to endothelial cells (11). We generated atx−/+ flk−/+ and atx−/− flk−/+ embryos with flk-1 promoter-regulated lacZ expression. To do this we first crossed atx−/− mice with flk−/+ (flk LacZ) mice and generated atx−/+ flk−/+ and atx−/− flk−/+ embryos. Then atx−/+ flk−/+ and atx−/− flk−/+ embryos were generated by crossing atx−/+ flk−/+ mice. At E8.5, atx−/− flk−/+ yolk sacs had slightly rough but almost comparable tubular structures to atx−/+ flk−/+ yolk sacs (Fig. 5B). In contrast, at E9.5, atx−/− flk−/+ yolk sacs were found to contain giant vacuoles and large ventral and cardiac surfaces. At E10.5 (Figs. 5C–E) and later, atx−/− flk−/+ embryos exhibited pericardial effusion and thinned out hearts. The defect in cardiac development was supported by the finding that embryos lacking ATX had pericardial effusion and many died at E10.5. Homozygous embryos survived at least to 10.5 days of gestation. Homozygous embryos had almost all of the defects found in homozygous mutant mice at earlier stages of development. These results indicate that ATX is crucial for development and survival from E8.5 to E10.5.

### Table 1

| Stage of development | Number of litters | Total number of embryos tested | Numbers of embryo or neonate | Embryo resorption |
|----------------------|------------------|-------------------------------|-------------------------------|------------------|
| E8.5                 | 7                | 65                            | 14 +/+ 35 +/− 16 −/−           | 0                |
| E9.5                 | 8                | 74                            | 19 +/+ 36 +/− 19 −/−           | 0                |
| E10.5                | 7                | 62                            | 22 +/+ 29 +/− 11 −/−           | 3                |
| E11.5                | 4                | 33                            | 11 +/+ 22 +/− 0 −/−            | 9                |
| E12.5                | 1                | 7                             | 4 +/+ 3 +/− 0 −/−             | 3                |
| E13.5                | 2                | 15                            | 3 +/+ 12 +/− 0 −/−            | 3                |
| E14.5                | 1                | 6                             | 3 +/+ 3 +/− 0 −/−            | 1                |
| Neonate              | 9                | 45                            | 15 +/+ 30 +/− 0 −/−           | −                |

### Figure 4

**Morphologies of atx+/+ and atx−/− embryo proper at E8.5, E9.5, and E10.5.** At E8.5 (A–C) about half of the atx+/+ embryos appear to be normal compared with atx−/− embryos (A and C), whereas the other half of embryos showed abnormal morphologies in head regions (B, arrow). At E9.5 (D–F) and E10.5 (G and H) atx−/− embryos are easily distinguishable from atx+/+ or atx+/− littermates. atx−/− embryos exhibit several defects such as pericardial effusion (F, arrow) and open and kinky neural tube (F, arrowhead). Scale bars, 100 μm in panels A–F and 200 μm in panels G and H.
Autotaxin Knock-out Mice

A

B

C

D

E

F
have severe vascular defects, i.e. they had no well developed fine blood vessels (Fig. 5B). Consistent with this observation, both normal and abnormal vessels were visible in atx<sup>-/-</sup> flk<sup>+/+</sup> yolk sacs at E9.0 (Fig. 5B). Embryos at E8.5 atx<sup>-/-</sup> had vascular defects in the head (arrows) and cardiac (sinus venosus) regions but not in the dorsal aortae (Fig. 5B). Staining with antibodies against PECAM, another endothelial cell-specific marker, confirmed the vascular defects of the yolk sac in homozgyous embryos (not shown). Incomplete vascular remodeling was also observed in the embryo proper. Whole-mount anti-PECAM staining revealed well-developed thin and fine tubule-like structures in atx<sup>+/+</sup> embryos (Fig. 5C). In sharp contrast, the structures were thick and irregular-shaped in atx<sup>-/-</sup> embryos (Fig. 5C). At E9.5, the atx<sup>+/+</sup> placenta had both embryonic and maternal blood vessels ( distinguishable by the size of their erythrocytes), whereas the atx<sup>-/-</sup> placenta had only maternal blood vessels (Fig. 5D). At E9.5, chorioallantoic attachment and fusion were observed both in atx<sup>+/+</sup> and atx<sup>-/-</sup> embryos (Fig. 5E). However, it was evident that atx<sup>-/-</sup> allantois lacked the numerous vessel lumen visible in the atx<sup>+/+</sup> allantois (Fig. 5E). Immunostaining of the yolk sac and embryo proper at E9.5 with anti-α-smooth muscle actin, a specific marker of vascular smooth muscle cells, showed that smooth muscle cells were present and surrounded the endothelial cells in both the yolk sac (Fig. 5F) and embryo proper (not shown). This indicates that mural cell investment is not the cause of vascular defects in atx<sup>-/-</sup> embryos. These analyses suggest that initial blood vessel formation occurs properly in atx<sup>-/-</sup> embryos but the newly formed blood vessels fail to develop into mature vessels in the absence of ATX.

RT-PCR experiments detected modest expression of ATX mRNA in both the yolk sac and embryo proper until E10.5 (Fig. 6A). In situ hybridization experiments detected weak expression in endodermal cells that surround the yolk sac at E8.5 (Fig. 6B). By contrast, significant ATX protein expression was observed in amniotic fluids in all embryonic stages tested, and

![Figure 5](image_url)

**Figure 5. Defects in vascular remodeling in atx<sup>-/-</sup> embryos.** A, defects in the yolk sac vasculature. Yolk sac from atx<sup>+/+</sup> and atx<sup>-/-</sup> embryos at E9.5 and E10.5. Sections are stained with hematoxylin and eosin E. Note the normal blood vessels (arrowheads) in atx<sup>+/+</sup> and their absence in atx<sup>-/-</sup> yolk sac. Arrows indicate that erythrocytes are present in atx<sup>-/-</sup> yolk sacs. E, erythrocytes. B, whole-mount LacZ staining of atx<sup>+/+</sup> flk<sup>+/+</sup> and atx<sup>-/-</sup> flk<sup>+/+</sup> embryos (E8.5) and yolk sacs (E8.5, E9.0, and E9.5). At E9.0 both normal and abnormal (arrowhead) vessels are visible in atx<sup>-/-</sup> yolk sacs, whereas only normal vessels are visible in atx<sup>+/+</sup> yolk sacs. DA, dorsal aorta; H, heart; VA, vitelline artery; SV, sinus venosus. C, whole-mount PECAM immunostaining of atx<sup>-/-</sup> and atx<sup>+/+</sup> embryos at E9.5. Magnifications of head regions are shown at right. D, sections of atx<sup>+/+</sup> and atx<sup>-/-</sup> placenta at E9.5 were stained with haematoxylin and eosin. Arrowheads, maternal blood vessels; arrows, embryonic blood vessels. E, histological analysis of allantoic development and vascularization. In atx<sup>-/-</sup> embryos, the allantois forms a large, erythrocyte-filled vessel connected to placenta at E9.5. atx<sup>-/-</sup> allantois is sparse and lacks the numerous vessel lumen visible in the atx<sup>+/+</sup> allantois. F, expression of α-smooth muscle actin-positive cells in atx<sup>+/+</sup> and atx<sup>-/-</sup> yolk sacs at E9.5. e, erythrocytes. Scales are indicated in each picture.
its level was nearly equivalent to the plasma level as judged by both Western blotting and lysoPLD activity (Fig. 2, A and C). As was observed in plasma, the lysoPLD activity and ATX protein in amniotic fluids from atx+/− embryos at E11.5, 12.5, and 13.5 were almost half the value found in atx−/− embryos (Fig. 2, A and C). Western blot analysis also showed that ATX expression was not detectable in either yolk sac or embryo proper at E9.5, although ATX protein was highly expressed in amniotic fluids (Fig. 2, D and E). These analyses indicate that most of the ATX proteins produced in embryos are released and concentrated in amniotic fluids.

**ATX and LPA Prevent Disassembly of Vessels in Allantois Culture System**—To determine the role of ATX and to assess its enzymatic activity in vascular remodeling, we utilized the allantois explant culture system (18). As reported by Argraves et al. (18), when E8.5 allantois explants were cultured in the presence of 10% fetal calf serum, a highly branched network of PECAM-positive vessels was visible after 24 h of culture (Fig. 7A). The amniotic fluids isolated from E12.5 wild-type embryos were found to have a similar but an even more pronounced effect on vessel formation than fetal calf serum (Fig. 7A). Allantois explants from atx−/− embryos also showed a highly branched network of PECAM-positive vessels when they were cultured in the presence of amniotic fluids isolated from E12.5 wild-type embryos (Fig. 7A) or fetal calf serum (not shown). Adding LPA or recombinant ATX to this system had little effect on vessel formation (Fig. 7A), showing that ATX and LPA themselves are not angiogenic. In the presence of amniotic fluids, the vessels that formed within the first 24 h of culture kept their tubular structure during the next 18 h of culture (Fig. 7B). However, in the absence of amniotic fluid, the preformed nascent vessels were unstable and disassembled (Fig. 7B). The recombinant ATX, but not catalytically inactive ATX, prevented the disassembly (Fig. 7B). LPA also prevented the disassembly of preformed vessels, whereas S1P had no effect (Fig. 7B). These results indicate that ATX in the amniotic fluids has a role in stabilizing vessels.

To know the molecular mechanisms of how LPA stabilizes blood vessels, we examined the expression of LPA receptors. RT-PCR and in situ hybridization experiments showed that among the four LPA receptors LPA₁ is predominantly expressed in the yolk sac and embryo proper at E8.5, E9.5, and E10.5 (Fig. 6C), where it is expressed by cells in the endodermal layer other than endothelial cells (Fig. 6D). Expression levels of

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**FIGURE 7. ATX and LPA stabilize preformed vessels and prevent endothelial disassembly in allantois explants.** A, anti-PECAM-labeled fluorescent microscopy images of E8.5 allantois cultured for 24 h in amniotic fluids isolated from E12.5 embryos or Dulbecco’s modified Eagle’s medium containing recombinant ATX (1 μg/ml), LPA (1 μM), and fetal calf serum (10%). Neither ATX nor LPA shows angiogenic activity. The amniotic fluids promote blood vessel formation of allantois isolated from atx−/− embryos. B, allantoises were cultured for 24 h in the presence of amniotic fluids to form blood vessels. Then, the vessels were cultured for 18 h in Dulbecco’s modified Eagle’s medium in the presence or absence of recombinant ATX (1 μg/ml), catalytically inactive mutant ATX (T209A mutant, 1 μg/ml), LPA (1 μM), S1P (1 μM), or amniotic fluids. ATX and LPA prevent endothelial disassembly and stabilize vessels in allantois explants. Experiments were performed at least three times in triplicate, and the representative results were shown.
Ki16425 did not show antagonistic activity toward LPA4 (19).

ATX was originally identified as a cell motility-stimulating factor for cancer cells (1). Later, ATX was found to have enzymatic activity to produce a bioactive phospholipid, LPA (5, 6, 20). LPA has been defined as a growth factor-like lipid with numerous biological activities, including stimulation of cell motility (3). In this study to elucidate the physiological role of ATX we analyzed ATX knock-out mice. To our surprise ATX knock-out mice were embryonic lethal, showing that ATX is indispensable for embryonic development. Blood vessel formation was severely affected in ATX-null embryos, suggesting novel roles of ATX and LPA in the formation of blood vessels in the embryonic stage. In addition, the present study confirmed that ATX is responsible for bulk LPA production in blood, but not for S1P production. The phenotype of ATX-null embryos was quite different from those of LPA receptors reported so far (21–23). These results raise a possibility that ATX and LPA contribute to embryonic blood vessel formation through as yet unidentified LPA receptors.

LPA$_{2}$, LPA$_{3}$, and LPA$_{4}$ were revealed to be low. Thus, we tested the effect of an LPA receptor antagonist, Ki16425, on the vessel-stabilizing effect of ATX and LPA. The $K_{i}$ values of Ki16425 are 0.25 $\mu$M for LPA$_{1}$, 5.6 $\mu$M for LPA$_{2}$, and 0.36 $\mu$M for LPA$_{3}$ (19). Ki16425 did not show antagonistic activity toward LPA$_{4}$ (19). Adding Ki16425 at 5 $\mu$M in the allantois culture system did not affect the vessel-stabilizing effects of ATX and LPA (Fig. 8). Thus, it is unlikely that LPA$_{1}$, LPA$_{2}$, and LPA$_{3}$ are involved in the vessel-stabilizing effect of ATX and LPA.

**DISCUSSION**

Nam et al. (24) reported that injection of Matrigel mixed with purified ATX into athymic nude mice resulted in new blood vessel formation within the plug and that ATX stimulated human umbilical vein endothelial cells grown on Matrigel to form tubules. From these results they suggested that ATX was an angiogenic factor. Our present data suggest that ATX itself is not angiogenic but it stabilizes preformed vessels through an unknown mechanism. Because Matrigel contains several factors that promote vessel formation, ATX may contribute to the vessel formation possibly in cooperation with such factors.

The formation of vasculature by vasculogenesis and angiogenesis is essential not only for embryonic development but also for the unrestricted growth of tumors (25). ATX stimulates both cell proliferation and cell motility of cancer cells through LPA production (5). In addition, overexpression of ATX is frequently associated with malignant tumors such as small cell lung cancer (26), renal cell cancer (27), hepatocellular carcinoma (28, 29), breast cancer (30, 31), Hodgkin lymphoma (32), thyroid cancer (33), and glioblastoma (17). Thus, ATX has been implicated in the progression of malignant tumors. In this study we showed that ATX has an additional role in blood vessel formation, possibly by stabilizing preformed blood vessels. The present study raises the possibility that ATX, in addition to stimulating proliferation and motility of tumor cells (5), contributes to the progression of tumors by stabilizing blood vessels in the vicinity of tumors.

ATX is capable of producing S1P because ATX also catalyzes a reaction to convert sphingosylphosphocholine to S1P (7). We showed in this study that ATX is a major producing enzyme for LPA, but not for S1P, in blood. It is accepted in recent reports that S1P is produced intracellularly from sphingosine through phosphorylation reaction mediated by two isozymes of sphingosine kinase (sphK) (sphingosine kinase 1 and sphingosine kinase 2) (34, 35). Interestingly, vascular defects were also observed in sphingosine kinase-null and S1P receptor-null embryos. Both sphk1$^{-/-}$, sphk2$^{-/-}$ double mutant embryos (36) and s1p$_{1}^{-/-}$, s1p$_{2}^{-/-}$, s1p$_{3}^{-/-}$ triple mutant embryos (37) died around E12.5, showing that the phenotypes are milder than that of atx$^{-/-}$ embryos. Thus, despite their similar structures, LPA and S1P have distinct synthetic pathways, targets, and functions.

Among the four LPA receptors reported so far, LPA$_{1}$ is predominantly expressed in embryos around E9.5, whereas LPA$_{2}$, LPA$_{3}$, and LPA$_{4}$ are weakly expressed (Fig. 6, C and D). We
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showed that Ki166245, an antagonist for LPA_{1}, LPA_{3}, and LPA_{3}, had little effect on vessel-stabilizing activity of ATX and LPA (Fig. 8). Thus, it is unlikely that LPA_{1}, LPA_{2}, and LPA_{3} are involved in this process. Consistent with this, phenotypes of LPA receptor knock-out mice are quite different from that of ATX knock-out mice. LPA_{1}, LPA_{2}, and LPA_{3} single and LPA_{1} and LPA_{2} double knock-out mice are reported to be viable (21–23). LPA also exerts its role through other targets such as GPR23/LPA_{4} (38). Very recently a novel G protein-coupled receptor, GPR92/LPA_{9}, was identified as a fifth cellular receptor for LPA (39, 40), which showed the highest expression in gut. LPA_{4} and LPA_{3} knock-out mice have not been reported so far. It is possible that ATX exerts its role in blood vessel stabilization through these newly identified LPA receptors via signaling pathways independent of classical LPA receptors (LPA_{1}, LPA_{2}, and LPA_{3}).

Acknowledgments—We thank Drs. Wouter H. Moolenaar and Masayuki Masu for sharing unpublished results.

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