Autotaxin (NPP-2), a Metastasis-enhancing Motogen, Is an Angiogenic Factor

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ABSTRACT

Autotaxin (ATX-NPP-2), originally isolated as a tumor motility-stimulating protein, has recently been shown to augment tumor aggressiveness. Specifically, atx-transfected, ras-transformed NIH3T3 cell lines have been shown to be more invasive, tumorigenic, and metastatic than mock-transfected ras-transformed control cells. In addition, the atx-transfected ras-transformed cell lines appeared to produce tumors that were much more hyperemic than those formed by appropriate control cells. This observation led to the present study, in which we demonstrate that ATX modulates angiogenesis both directly and indirectly. We have used a murine in vivo angiogenesis model in which treated Matrigel plugs are injected s.c. into athymic nude BALB/c mice. Using the same transfected cell lines as before, we found that mixing atx-transfected ras-transformed NIH3T3 cells into the Matrigel resulted in greater new blood vessel formation than control cells. Similarly, mixing purified ATX into the Matrigel resulted in new blood vessel formation within the plug, similar to that produced by vascular endothelial growth factor. Mechanistically, ATX is not a strong chemotactrant for human endothelial cells (HUVECs); however, it strongly stimulates motility in human coro

INTRODUCTION

Cancer metastasis involves a number of concurrent and sequential processes. These include local invasion and migration of cells from the primary locus into a secondary site, vigorous proliferation of cells, and establishment of a vascular network to nourish the expanding population of cells (1, 2).

ATX, originally isolated as a tumor motility-stimulating protein (3), is a member of the nucleotide pyrophosphatase and phosphodiesterase family of exo- and ecto-enzymes (4, 5). The intact phosphodiesterase active site is necessary for its motility-stimulating activity because a single point mutation (T210A) abolishes both activities (6). In addition, we have recently demonstrated that ATX enhances tumor aggressiveness (7). This was accomplished using ras-transformed NIH3T3 cells that had been stably transfected with human ATX cDNA. These cells demonstrated increased invasiveness, tumorigenicity, and metastatic potential compared with appropriate controls. In the course of these experiments, we repeatedly observed that tumors produced by injecting the atx-transfected ras-transformed cells into athymic nude mice were much more hyperemic than mock-transfected, ras-transformed controls. That observation led us to assess whether ATX could play a role in new blood vessel formation.

Induction of angiogenesis is essential for continuous growth of primary neoplasms, as well as for the establishment of metastatic nidi (8). Like motility and metastasis, angiogenesis is a complex process requiring a coordinated series of events including endothelial cell proliferation, migration, tube and lumen formation, and, in some cases, recruitment of smooth muscle and other adventitial cells (9). In the present study, we use both atx-transfected tumor cells and the purified ATX protein to quantify its role in angiogenesis.

MATERIALS AND METHODS

Cells. Normal HUVECs were purchased from Clonetics (San Diego, CA) and maintained in EBM-2 supplemented with the Clonetics Bullet Kit, which includes a preselected fetal bovine serum, human recombinant epithelial growth factor, human bFGF, VEGF, and human recombinant IGF-I. Human CASMCs and human aortic smooth muscle cells were also purchased from Clonetics and were maintained in smooth muscle growth medium 2 supplemented with 0.5 ng/ml epithelial growth factor, 0.5 ng/ml insulin, and 0.5 ng/ml bFGF. All cells were maintained at 37°C in 5% CO2 and 95% ambient air, and culture medium was changed every other day. Biological experiments were performed with cells between the third and sixth passages.

ATX-overexpressing clones (3T3-Ras-ATX cell lines, ras-transformed NIH3T3 cells transfected with ATX cDNA) and a control clone (3T3-Ras-Mock, ras-transformed NIH3T3 cells transfected with empty plasmid vector) were obtained by transfecting ras-transformed NIH3T3 (clone 7) cells with atx-transfected expression vector or empty vector, respectively (7). These transfected cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and 2 mM l-glutamine.

Cell Motility Assay. Migration assays of HUVECs and CASMCs were performed in 48-well modified Boyden chambers (Neuroprobe Inc., Cabin John, MD) as described previously (10). Cells were harvested using a trypsin/EDTA solution (Clonetics) and resuspended in appropriate basal medium. The bottom wells were filled with varying concentrations of purified ATX (5) or 10 ng/ml human recombinant VEGF (positive control). Upper and lower chambers were separated by gelatin-coated polycarbonate filters with 8 μm pores (Neuroprobe, Inc.). Cells (5 × 10⁴) were placed into each upper well, and then the chamber was incubated for 4 h at 37°C in a 5% CO₂ humidified incubator. The membranes were fixed and stained with Diff-Quik (Baxter Scientific, McGraw Park, IL) and mounted on a glass slide. Cell migration was performed in triplicate and quantified under light microscopy by counting cells in five randomly chosen HPFs.

Analysis of Growth Factor-induced ATX mRNA Expression in HUVECs. The expression of ATX mRNA after growth factor treatments was determined using RT-PCR with ATX-specific oligonucleotide primers (sense primer, 5'-CTCCTCATCTAGTGTCCTAAATAGG-3'; antisense primer, 5'-CTTACAGGATGGTGAGCAATCTTGC-3'). After 18–24 h of starvation in the appropriate unsupplemented EBM-2, cells were treated with media containing the indicated growth factor(s) for 1 h. Total cellular RNA was isolated from confluent cell cultures by using the Micro RNA Isolation Kit (Stratagene, La Jolla, CA) and reverse-transcribed into cDNA using GENE AMP RNA PCR Kit (Perkin-Elmer, Foster City, CA) following the manufacturer’s suggested protocol. PCR amplification was performed as...
described previously (6). The relative amounts of mRNA were normalized to internal β-actin controls.

Western Blot Analysis. HUVECs were starved in unsupplemented EBM-2 for 24 h, and then the medium was replaced for an additional 24 h with EBM-2 supplemented only with the indicated growth factor(s). Each conditioned medium was collected, concentrated, and partially purified using agarose-bound concanavalin A (Vector Laboratories, Burlingame, CA; Ref. 11). For each partially purified conditioned medium, 25 µg of total protein were loaded and separated on 8–16% SDS-PAGE, and ATX was detected with anti-ATX polyclonal antibody as described previously (4).

Matrigel Microtubule Forming Assay. To determine whether ATX induced in vitro angiogenesis, HUVECs were grown on Matrigel-coated 96-well plates as described previously (12), with the following modifications. Matrigel is known to contain a variety of growth factors (13). To limit spontaneous angiogenesis, growth factor-reduced Matrigel (Becton Dickinson, Bedford, MA) was added to the wells of 96-well plates and incubated at 37°C for 30 min to allow it to gel. HUVECs were starved for 18–24 h in unsupplemented EBM-2 before being harvested, and then 1 × 10^4 HUVECs were resuspended in fresh medium, added to triplicate wells, and allowed to adhere to the gels for 1 h at 37°C. After that time, media were replaced with EBM-2 supplemented with 30 ng/ml recombinant VEGF or varying concentrations of ATX as indicated. After 18 h, the plates were fixed, stained with Diff-Quik, and photographed.

In Vivo Angiogenesis in Matrigel Plugs. To examine the in vivo effects of ATX expression on tumor angiogenesis, we used the Matrigel plug model system (14), using previously established human atx-transfected ras-transformed cell lines (7). Briefly, 2.5 × 10^4 cells were mixed with liquefied Matrigel (final volume, 0.5 ml) and injected s.c. into the flanks of 6–7-week-old female (BALB/c) athymic nude mice. Controls received an injection of either Matrigel alone or Matrigel mixed with empty vector-transfected cells. Animals were sacrificed after 10 days, and the Matrigel plugs were recovered and photographed. The plugs were then fixed and embedded in paraffin, sectioned and mounted onto glass slides, and stained with H&E for histological analysis.

Histology and Immunohistochemical Staining. Specimens, including Matrigel plugs, were fixed overnight in 10% buffered formalin, dehydrated, and embedded in paraffin wax. Sections were stained with H&E and examined with light microscopy. Immunohistochemical staining for the presence of CD31 and α-SMA anti-

![Fig. 1](Image URL)
Highly Vascular Tumors. Previously, we demonstrated that ATX, a Ras homolog, stimulates angiogenesis in vitro and enhances tumor growth and metastatic potential in vivo (7). These observations were made using two transfected cell lines derived from ras-transformed NIH3T3 (clone 7) cells (3T3-Ras); (a) empty vector-transfected, ras-transformed cells (3T3-Ras-Mock cells); and (b) atx-transfected, ras-transformed cells (3T3-Ras-ATX cells). We also noted that the tumors produced by the 3T3-Ras-ATX cells were very highly vascular. This observation led us to perform additional experiments to determine what role ATX might play in blood vessel formation. We used an experimental murine angiogenesis model system in which ras-transformed NIH3T3 (3T3-Ras), 3T3-Ras-Mock, or 3T3-Ras-ATX cells were mixed with Matrigel and injected s.c. into athymic nude mice. The Matrigel plugs were removed 10 days after inoculation and evaluated for angiogenesis. Whole plugs injected with 3T3-Ras-ATX cells showed greater vascularity compared with those injected with 3T3-Ras-Mock cells (Fig. 1, A and B, respectively). Light microscopic analysis of H&E-stained sections from these Matrigel plugs (Fig. 1, C–F) revealed the formation of canalized linear and cross-sectional vessels in all of the plugs. However, the 3T3-Ras-ATX-injected plugs also had extensive microaneurysms filled with RBCs (Fig. 1, E and F). In addition, these same 3T3-Ras-ATX Matrigel plugs appeared to contain more vigorously growing tumor cells, which were seen as cords and clusters of darkly stained fibroblastic cells growing from the edge of the Matrigel plug inward. Detailed pathological, histomorphological analysis indicates that an inflammatory infiltrate is slight to none.

Because we had previously shown that the growth rates of 3T3-Ras-Mock and 3T3-Ras-ATX cells were essentially identical under tissue culture conditions (7), these differences in tumor growth in vivo could be attributed to augmented blood vessel formation in the presence of ATX.

Effects of ATX on HUVECs and Arterial Smooth Muscle Cells in Vitro. Because ATX is a potent tumor motogen, it seemed possible that ATX might affect angiogenesis by chemotactically stimulating human endothelial or arterial smooth muscle cells to move into a forming metastatic nidus. When ATX was presented as chemotaectant to HUVECs (Fig. 2A), it stimulated a weak response that had, at best, borderline significance compared with control assays with medium in the lower well of the chamber. Concurrent assays run with VEGF as attractant demonstrated significant motility (P < 0.001 compared with controls, as determined by a nonpaired, two-tailed t test) greater than twice that of unstimulated controls. In contrast, CASMCs (Fig. 2B) had a significant motility response to ATX (P < 0.01 compared with controls, as determined by nonpaired, two-tailed t test). Aortic artery smooth muscle cells demonstrated similarly significant motility in response to ATX (data not shown). Because this motility result was unexpected, the experiment was repeated with human skin and lung microvascular endothelial cells. Preliminary results show a pattern that is nearly identical to that of the HUVECs. ATX evokes a weaker motility response than VEGF (data not shown).

Formation of tubules by HUVECs grown on Matrigel is another in vitro predictor of angiogenic potential. HUVECs were starved for...
18–24 h, layered onto growth factor-depleted Matrigel with or without ATX or VEGF, and allowed to grow overnight. Most of the control cells, maintained in unsupplemented EBM-2, exhibited a small round shape and did not spread, although a few formed rather truncated tubules (Fig. 3A). Similar results have been described previously (15). In the presence of VEGF (Fig. 3B), the cells formed long capillary-like tubular arrays across the Matrigel. The addition of ATX (Fig. 3C and D), especially at the higher (1 nM) concentration tested (Fig. 3D), resulted in tubules much like those seen with VEGF.

Because both HUVECs and the arterial smooth muscle cells responded to the presence of external ATX, we next examined whether either type of cell expressed the protein. The highly sensitive RT-PCR amplification assay was chosen to detect ATX mRNA using human cells from different patients or donors. In all experiments (data not shown), HUVECs from three of three donors were positive, as were five of five patient CASMCs and two of two patient aortic smooth muscle cells.

ATX Protein Stimulates Blood Vessel Formation in Matrigel Plugs. To determine whether ATX itself was responsible for the angiogenic effects we had seen in atx-transfected cell lines, we next assayed the capacity of the recombinant protein to stimulate new blood vessel formation in the in vivo Matrigel plug assay. ATX (1 nM)
was mixed with growth factor-depleted Matrigel and injected s.c. into athymic nude mice. VEGF (30 ng/ml) and Matrigel alone served as positive and negative controls, respectively. The plugs were recovered either 6 or 12 days after injection, formalin-fixed, paraffin-embedded, and stained with H&E or immunostained with anti-CD31 or anti-α-SMA antibodies. As shown in Fig. 4A, the growth factor-depleted Matrigel plug resulted in very sparse blood vessel formation. In contrast, VEGF resulted in obvious blood vessel formation by day 6 (Fig. 4B), which was closely associated with adipogenesis, as has been noted previously (16). ATX also resulted in prominent blood vessel formation (Fig. 4C), although the lipogenic effect was slightly delayed compared with that of VEGF. The presence of endothelial cells in proliferating capillaries was confirmed in both the VEGF- and ATX-treated plugs by immunohistochemical staining for a specific endothelial marker, CD31 (Fig. 4, E and F, respectively). Immunohistochemical staining for α-SMA confirmed that α-SMA-positive pericyte-like cells surrounded the endothelial cell layer in the same treatment groups (Fig. 4, G–I). Histologically, the pericyte-like cells were noted to be more spindle-shaped and were localized distally from the capillary lumen compared with endothelial cells. These data suggest that ATX might contribute to the maturation of newly formed capillaries.

We quantified these results by counting only microvessels that contained RBCs within their lumen under light microscopy (Fig. 5). The day 6 and day 12 Matrigel controls had nearly identical averages of 1.7 and 2.6 vessels/HPF, respectively. At day 6, the ATX and VEGF plugs had averages of 13.6 and 14.8 vessels/HPF, respectively. These values were not significantly different from each other, but they were significantly different from the day 6 Matrigel controls (P < 0.0001 for both, using unpaired, two-tailed t test). Similarly, by day 12, ATX-treated plugs had 24.2 vessels/HPF (P < 0.0001 compared with vessels in day 12 Matrigel control). Based on these results, we conclude that ATX directly stimulates new blood vessel formation and appears to be a novel angiogenesis-stimulating factor.

Effect of Growth Factors on ATX Expression and Secretion by HUVECs. HUVECs were grown in serum-free EBM-2 supplemented with a variety of growth factors known to sustain their growth. These included bFGF, VEGF, IGF-I, and epithelial growth factor. Under these conditions, the HUVECs were found to express abundant ATX. Because little is known about regulation of ATX expression and secretion or about how ATX could fit into the complicated process of angiogenesis, we tested the individual growth factors in the medium to determine whether any of them affected ATX. HUVECs were starved overnight in unsupplemented EBM-2 and then fed with fresh medium supplemented with individual growth factors for 1 h. All four factors were added to EBM-2 as a positive control, and unsupplemented EBM-2 served as a negative control. ATX expression was tested by RT-PCR amplification and compared with expression of the internal control, β-actin. Treatment with bFGF appeared to stimulate ATX expression to levels equivalent to positive controls. Other growth factors appeared to have no effect (Fig. 6A). In contrast, homologous assays performed with CASMCs demonstrated that none of the tested growth factors affected ATX expression (data not shown). Time course studies of the bFGF effect on ATX expression revealed maximal stimulation after approximately 1 h of exposure (data not shown).

We next determined whether bFGF also affected secretion of ATX by endothelial cells. Conditioned media were prepared for each growth factor treatment, partially purified by concanavalin A lectin affinity, and concentrated. Proteins were separated by gel electrophoresis and analyzed by Western blot using ATX-specific anti-peptide antibodies. As seen in Fig. 6B, bFGF treatment resulted in a secretion of ATX equivalent to that of the positive control. No other

![Figure 5](image55x552 to 286x741)

**Fig. 5.** Quantification of microvessels in Matrigel plugs. Microvessels were quantified in triplicate Matrigel plugs excised from mice 6 or 12 days after injection with Matrigel alone, Matrigel mixed with 30 ng/ml VEGF, or Matrigel containing 1 nM ATX. Only vessels with patent lumens containing RBCs were counted in quadruplicate HPFs (>200) under light microscopy. Bar, mean value for each experimental group.

![Figure 6](image352x136 to 517x471)

**Fig. 6.** Effect of growth factors on cellular expression and secretion of ATX. A, ATX mRNA levels were assessed for HUVECs grown in the absence of serum and in the presence of different growth factors. Confluent starved cultures of HUVECs were exposed to each indicated growth factor for 1 h. For each treatment group, 1 μg of total RNA was analyzed by RT-PCR as described in “Materials and Methods.” The relative amounts of mRNA were normalized to internal β-actin controls. B, confluent cultures of HUVECs were exposed to each indicated growth factor for 18 h. Growth factor-treated conditioned media were concentrated and partially purified, and samples were analyzed by Western blot. C, the 5′ nucleotide phosphodiesterase (PDE) activity in growth factor-treated conditioned media was measured using the p-nitrophenyl-TMP colorimetric assay.
treatment resulted in detectable secretion of ATX. Each of these partially purified media was also tested for phosphodiesterase activity (Fig. 6C). Media from positive-control and bFGF-treated cells produced greater enzyme activity than media from other treatment groups.

Thus, bFGF appears to increase the expression and secretion of active ATX protein by HUVECs, but not by CASMCs. bFGF itself has angiogenic properties, which raises the possibility that it could mediate part of its effect through the induction of ATX expression.

**DISCUSSION**

We had observed previously that atx-transfected, ras-transformed cells, when infused i.v. or injected s.c. into nude mice, produced markedly hyperemic tumors compared with empty vector-transfected, ras-transformed control cells (7). In this study, we provide evidence that the potent tumor motogen ATX is also an angiogenic factor. Using an in vivo murine angiogenesis model that employs s.c. Matrigel plugs, we provide evidence that the same atx-transfected, ras-transformed cells elicit a stronger angiogenic response with more rapid cellular proliferation than that elicited by control cells. Furthermore, using this same model system with purified ATX protein, we quantified the angiogenic response and found it comparable to that elicited by VEGF. Thus, the ATX protein alone appears to induce new blood vessel formation at concentrations similar to those that induce in vitro motility and invasion.

To elucidate the mechanisms by which ATX might affect new blood vessel formation, we grew HUVECs on a Matrigel substratum exposed to ATX and found that microtubule formation was significantly stimulated. Whereas this phase of blood vessel formation is crucial, the migration of the growing sprout of vasculature into the tumor is certainly an important feature of the process. However, we did not observe a significant in vitro effect of ATX on endothelial cell motility when compared with the potent chemotactrant VEGF. This was true for three tested groups of endothelial cells: (a) HUVECs; (b) lung microvascular cells; and (c) skin microvascular cells. This was certainly an unexpected result, and it suggests that the motility and angiogenic properties of the ATX molecule could be regulated separately. It is also conceivable that ATX may induce local expression of angiogenic factors such as bFGF and VEGF in vivo, which could have a direct effect on endothelial cell motility. In contrast, arterial smooth muscle cells, which are important to the maturation of newly formed blood vessels, did respond to ATX as a chemoattractant. It would be intriguing to know whether pericytes are similarly sensitive to ATX-stimulated motility. These data imply that ATX is more tubulogenic than motogenic in the early stages of angiogenesis.

Further insight into the complexity of the role ATX appears to play in new blood vessel formation was provided by experiments demonstrating that the angiogenic cytokine bFGF up-regulated expression and secretion of ATX by HUVECs. In contrast, bFGF had little effect on ATX expression in arterial smooth muscle cells. Furthermore, other tested angiogenic factors, such as VEGF, IGF-1, and epithelial growth factor, had no demonstrable effect on ATX expression in either cell type. Because HUVECs and arterial smooth muscle cells are shown to express ATX, these results suggest that both autocrine and paracrine regulatory mechanisms may be at work in ATX stimulation of new blood vessel formation. It is also possible that bFGF might mediate some of its angiogenic effects through its induction of ATX.

Angiogenesis is a multistep process that is initiated by degradation of basement membrane-surrounding capillaries, followed by the invasion of the underlying endothelial cells into the stroma. These endothelial cells proliferate and form cords of cells that develop lumens to become immature blood vessels (17). A process of maturation then begins (18) with the migration of pericytes around the vessels and the formation of a mature basement membrane. This is followed by arteriogenesis, in which the vessels become surrounded by a coat of smooth muscle cells, enabling the vessels to exhibit the vasomotor properties of mature arterioles. A number of cytokines have been shown to stimulate different stages of this process. Both VEGF and bFGF are important in the early stages of vasculogenesis and angiogenesis. Matrix metalloproteinases, which are released by endothelial cells and other cells including tumor cells, are matrix-degrading enzymes that could facilitate the migration of the growing vascular sprout. They have been found to bind to the endothelial cell surface and to initiate signal transduction pathways that in turn mediate detachment from basement membrane, proliferation, migration, and tubulogenesis. (19, 20). Our data suggest that ATX has a minimal direct effect on HUVEC migration but does affect HUVEC tubule formation and smooth muscle cell migration. These observations, along with the fact that bFGF stimulates ATX expression, suggest that ATX may exert its effects slightly later than bFGF in angiogenesis and may act in concert with other angiogenic factors to facilitate new blood vessel formation.

ATX, which was initially isolated as a tumor motility-stimulating protein, has now been shown to have angiogenic properties. It has been found to be expressed in melanoma (3), teratocarcinoma (6), and neuroblastoma (21) cell lines. In addition, its expression is greater in hepatocellular carcinoma than in normal liver tissue (22), and it is overexpressed in poorly differentiated non-small cell lung cancer (23). In previous studies with atx-transfected, ras-transformed cells, we demonstrated that although ATX does not by itself appear to confer oncogenicity, it does augment in vitro invasiveness as well as in vivo metastatic potential and tumor cell proliferation (7). These latter findings now appear to be partially explained by the angiogenic effect of ATX. As both a tumor motogen and an angiogenic factor, ATX appears to have the capacity to stimulate multiple facets of the metastatic cascade. It may accomplish this by providing a microenvironment conducive to invasiveness for normal endothelial cells and tumor cells.

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