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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Autotaxin (ATX) is a tumor cell-derived enzyme that regulates cancer cell growth motility and survival. ATX exhibits both phosphodiesterase and lysophospholipase D (lysoPLD) catalytic activities. The lysoPLD activity of ATX has been reported to generate lysophosphatidic (LPA) acid via hydrolysis of lysophosphatylcholine substrate. LPA exerts its effects on target cells primarily through the actions at G-protein coupled cell surface receptors. Because the actions of ATX on cancer cells are very similar to those elicited by LPA, the biological activity of ATX has been suggested to result from the production of LPA by the hydrolysis of lysoglycerophospholipid substrates that are produced by cells or present in cell culture systems. The broad purpose of the proposed research is to investigate the effects of LPA signaling in breast cancer cells. Specifically by targeting an important enzyme involved in the extracellular production of this bioactive lipid mediator. The long-term goal of the particular research presented in this report is to define the regulation, mechanism and biological significance of the lysophopholipase D activity of ATX.

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INTRODUCTION

Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are members of a class of lysophospholipid mediators that act through specific cell surface receptors to control a number of pivotal cellular processes[1]. Cultured breast cancer cell lines and primary breast cancer cell lines express receptors for LPA and S1P. There is credible evidence that breast cancer cells are responsive to LPA and S1P, that LPA and S1P may interact with growth factor receptor initiated signaling pathways[2,3,4], and that LPA and perhaps also S1P are synthesized by and/or in the vicinity of breast cancer cells. Accordingly, we proposed that targeting signaling actions of LPA would be beneficial in treatment of the disease. To this end, we have targeted one of the enzymes, called autotaxin (ATX) known to participate in the biosynthetic pathway for LPA, and more recently for S1P[5].

The tumor cell motility factor autotaxin has lysophopholipase D activity that can generate LPA by the hydrolysis of lysophosphatidylcholine[6]. Autotaxin (ATX) belongs to a broader family of integral membrane extonucleotide pyrophosphatase/phosphodiesterases (ENPPs) encoding structurally homologous proteins containing a nuclease and phosphodiesterase domain. Two additional genes are related to this family which lacks the nuclease and somatomedin domains[7]. Previous work supports the model that ATX is generated by proteolysis of the integral membrane precursor protein[8]. We have also been able to recapitulate this observation in a number of diverse cell lines, whereby a V5epitope-tagged variant is processed into a catalytically active soluble fragment. The soluble protein can be detected in cell lysates and is abundant in culture medium. Furin, a protease active in the secretory pathway, has an RXK/RR consensus site that is uniquely present in ATX at the expected location. Catalytic activity of the soluble form of ATX is significantly greater in the medium than within the cell. This suggests that processing of the protein is a critical regulatory step and may play a role in supporting the processivity of the enzyme.

ATX has been reported to have a stimulatory affect on cancer cell migration, growth and survival[9]. Since these responses can also be elicited by LPA it seems likely that many of the cellular affects of ATX can be attributed to its generation of LPA via its lysoPLD activity.

ATX mRNA has been reported to be more highly expressed in breast cancer cells than in normal breast epithelia. MDA-MB-435S with elevated endogenous ATX levels showed increased invasiveness as compared with MCF-7, MDA-MB-231, and HBL-100 cells. In addition, MCF-7 cells overexpressing ATX were reported to have increased motility and invasiveness as compared to vector controls[10]. Due to its ability to promote motility, ATX has been classified as a metastasis activator[11]. Therefore, breast cancer cells are a highly relevant system to investigate the biological actions of ATX, which we hypothesize to involve LPA.

BODY

The original proposed work was designed to study the effects of LPA signaling on growth, motility and survival in a breast cancer cell model system with the potential to target LPA receptors in therapeutics in order to modulate the effects of LPA on breast tumor cells. My specific area of interest and research focus was to characterize an important enzyme responsible for the extracellular generation of LPA (see figure 1) so as

to expose another potential therapeutic target that, by inhibition, could perhaps reduce cancer cell exposure to this pathologically beneficial lysolipid.

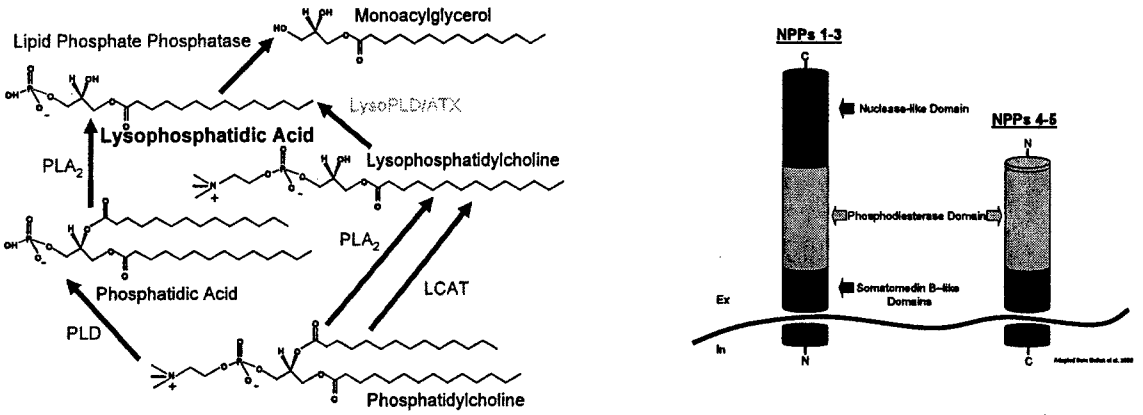


Figure 1. Pathways for LPA generation and degradation

Our first task was to transiently express V5-tagged ATX in various cell lines including T47D (Figure 2A) and MDA-MB-231 breast carcinoma cells (not shown) to examine whether or not they would produce the soluble proteolytically processed form of the protein. Both cell lines successfully generated significant amounts of soluble ATX in the culture medium. RT-PCR was conducted to look at endogenous levels of ATX mRNA. Data is not shown for the T47D cells because it needs to be optimized in this cell type. We also wanted to assess the localization pattern of ATX in the T47D cells via immunofluorescence and the protein localizes in the perinuclear region and dispersely throughout the cytoplasm. This pattern is likely indicative of ATX trafficking from ER to PM.

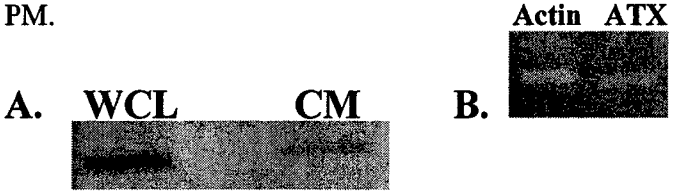


Figure 2. Expression of autotaxin.
A. T47D cells were transiently transfected with pCDNA3.1/GS-NPP2. Immunoblots probed with αV5 monoclonal antibody (ATX~125 kDa). WCL-whole cell lysate; CM-culture medium ***B.*** *RT-PCR of endogenous ATX in NIH3T3 cells* ***C.*** *Localization of ATX in T47D cells Immunofluorescence done using αV5 monoclonal antibody with goat anti-mouse Alexa488 secondary antibody.*

Since we were able to demonstrate proteolysis and secretion of ATX in these cells, our next question was to investigate which protease is responsible for conducting this cleavage. We took a candidate approach and identified furin as our first target

because it is a protease that is highly active in the secretory pathway going from the Golgi to the plasma membrane and sequence analysis revealed that ATX contained the furin consensus sequence just outside the transmembrane domain where the proteolysis is predicted to occur (Figure 3B). As shown in Figure 3A, ectopically expressed ATX is confirmed to co-localize with furin. The next approach utilized site-directed mutagenesis to alter the proposed furin consensus site with the expectation to prohibit production of soluble ATX. However, mutagenizing integral residues in this proteolysis site did not affect the processing of ATX and as shown in Figure 3C, soluble ATX in the culture medium remained unaffected. A time course comparing expression in the culture medium of ATX mutants with wild type ATX further confirmed that mutagenesis had no affect on production of soluble ATX (data not shown). We therefore, concluded that there was likely another protease recruited for proteolysis upon ablation of the furin site. Further experiments are necessary to address this possibility.

A.

B. Furin Consensus Cleavage Site: **-R-X-K/R-R-**

NPP1 72 TYKVLSLVLSVCVLTTILGCIFGLKPSCAKEVKSCK
NPP2 9 SCQIISLFTFAVGVSICLGFTAHRIKRAEGWEEG
NPP3 20 KYKIACIVLLALLVIMSLGLGLGLGLRKLEKQSCRK

C.

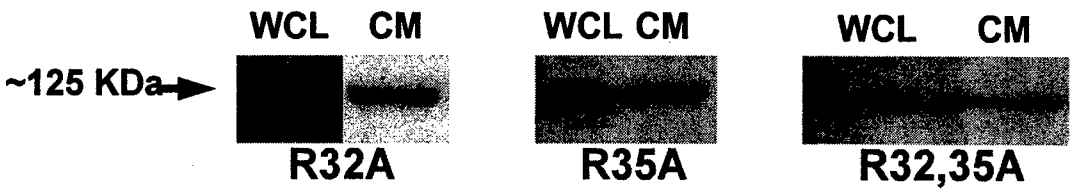
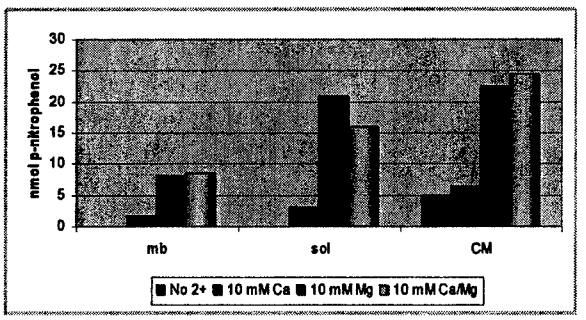
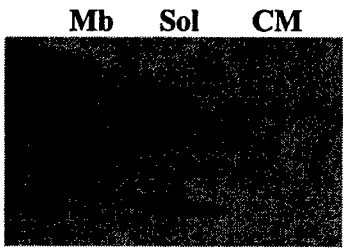


Figure 3. Furin: Protease Responsible for Autotaxin Proteolysis? A. *Hela cells transfected with pCDNA-ATX and co-stained with a monoclonal αV5 primary antibody and a polyclonal antibody to endogenous furin convertase followed by an Alexa488 and Alexa568 conjugated secondary anitbody, respectively.* ***B.*** *Sequences of NPP family members (transmembrane domain in blue)* ***C.*** *Expression of site-directed furin mutants.*

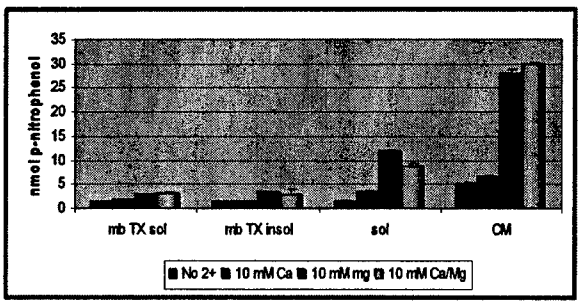
Because there are likely diverse roles for the membrane bound and the soluble form of ATX, we proposed that this could be a potential mode of regulation for its activity. Uncontrolled ATX activity could lead to constitutive production of and exposure to LPA, and if left unchecked would be detrimental to the cell. Therefore, the following experiments were designed to determine if differential activities could be attributed to the two forms of the enzyme. First the phosphodiesterase activity of ATX was examined under varying assay conditions. The results indicate that the soluble enzyme has 4-5 times more relative PDE activity than the membrane-bound form.

A.





B.



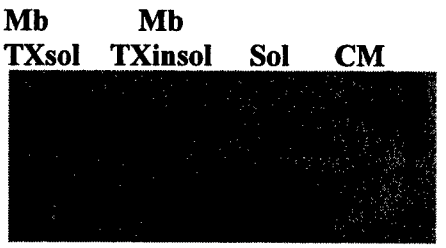
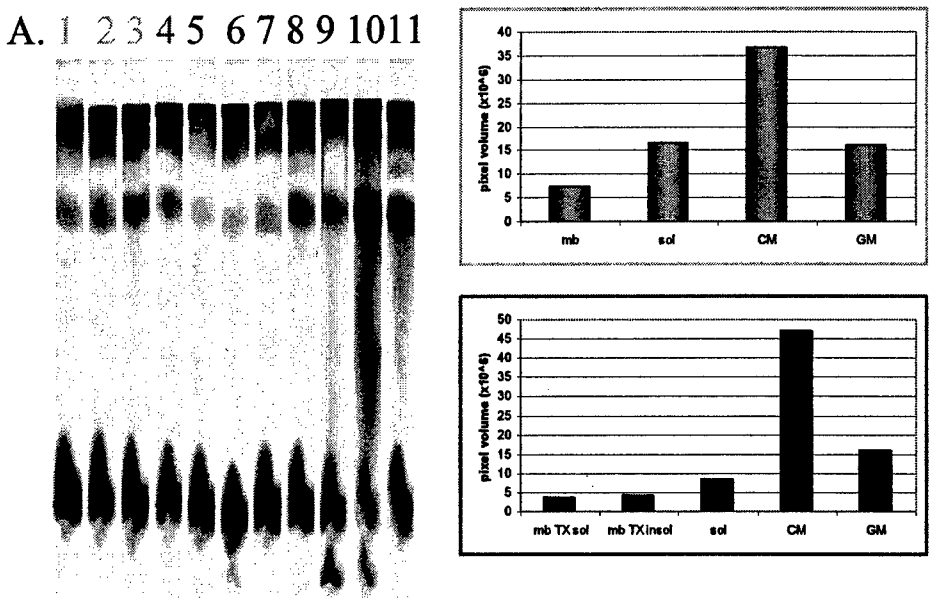


Figure 4. Phosphdiesterase Activity of Membrane vs. Soluble Autotaxin
SKOV3 cells stably expressing ATX were collected and fractionated. ***A.*** *The experiment outlined in yellow represents a comparison using a crude membrane preparation.* ***B.*** *The experiment outlined in blue utilizes Triton-X 100 soluble and insoluble membrane fractions. PDE activity is assessed using a chromogenic substrate (5 mM pNP-TMP) diluted in 100 mM Tris pH 8.9 and measuring the release of p-nitrophenol by reading A_{410}. To the right of the graphs are Western blots using the αV5 antibody.*

In conjunction with the experiments mentioned above, we wanted to look specifically at the lysophospholipase D activity of ATX with an assay system, adapted by our lab from methods used by Aoki et al., which utilizes a fluorescent lipid substrate and TLC. Likewise in these experiments lysoPLD activity was highest in the culture medium containing proteolytically processed ATX.



1. mb 2. sol 3. CM **4. GM 5. mb TXsol 6. mb TXinsol 7. sol 8. CM 9. –PLD 10. +PLD**

Figure 5. LysoPLD Activity of Membrane vs. Soluble Autotaxin
Samples described in Figure 5 were assayed using a fluorescently labeled substrate (NBD-LPC) and assessed based on their ability to generate LPA. As a control (lanes 10, 11), bacterial PLD is incubated with NBD-LPC to generate NBD-LPA. Samples are incubated for 90 min at 37° C in the presence of TX100 micelles containing the fluorescent substrate. The reactions are stopped and the lipids extracted using a modified Bligh and Dyer method. The fluorescent lipids are then separated by TLC and fluorescence is measured using a phosphor imaging instrument. The graphs on the right are the quantitative representation of this data.

We have also recently implemented a cell-based assay system that assesses chemotactic migration of cells to specific soluble agonists. In particular we possess the ability to observe and quanitate directed migration of cells toward autotaxin using fluorescent labeling of cells in our modified Boyden chamber assay. However, this experiment has not been conducted using the breast cancer cells, but is easily adaptable for any cell type.

Additionally, we have invested significant time and effort into developing a reliable system whereby we could express and purify large amounts of active ATX protein. This has proved to be more difficult than would be expected, despite concerted efforts with multiple methods including the baculovirus expression system, stable expression in High Five cells and FPLC purification from stably expressing mammalian cells. Purified protein would be crucial for future experiments designed to better characterize the enzyme properties of this protein as well as for biological assays looking

carefully at the pathophysiological effects of ATX on proliferation, apoptosis, migration and invasion.

Our model for the actions of ATX is represented in Figure 6. We hypothesize that plasma membrane bound ATX could move along the PM in a processive way catalyzing the hydrolysis of LPC substrate to generate extracellular pools of LPA which could then function in either an autocrine or paracrine manner to signal the tumor cell(s) to migrate, proliferate, and/or survive. Alternatively, soluble ATX located in the medium outside the cell could act on substrate that is present either on neighboring cell membranes or in microvesicles known to be released from various types of cancer cells including breast cancer cells (data not shown from our lab).

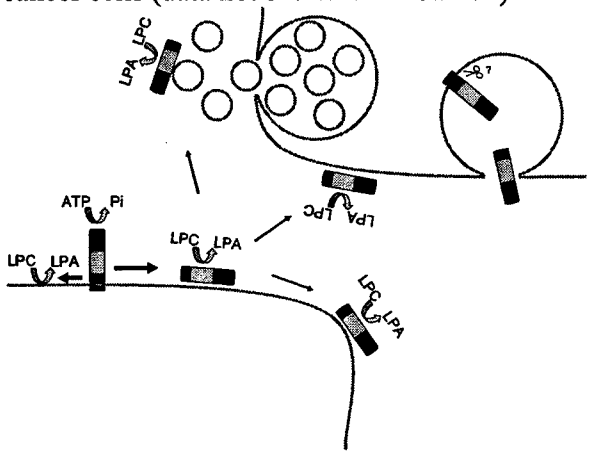


Figure 6. ***Model for the Processive Actions of the Soluble Catalytic Domain of NPP2.***

Future work:

- Investigate exactly which protease(s) are responsible for ATX processing
- Elucidate the mechanism of regulation for ATX activity.
- Determine if ATX is released from the cell surface or generated intracellularly by the secretory pathway and then released.
- Determine if ATX has a normal physiological or pathophysiological role in LPA generation.
- Assess whether or not the biological effects of ATX on cells can be wholly or partly accounted for by the generation of LPA.

KEY RESEARCH ACCOMPLISHMENTS

- Expression and localization of autotaxin in breast cancer cells
- Optimized RT-PCR protocol to readily detect endogenous ATX mRNA in various cell types
- Observed proteolytic processing of autotaxin in breast cancer cells
- Tested furin as the potential secretory protease responsible for autotaxin processing.
- Examined regulation of autotaxin by intracellular proteolysis and found that both lysoPLD and PDE activity is greater in the soluble fractions as compared to the membrane bound fractions
- Set up modified Boyden chamber assay for testing chemotactic migration of cells to autotaxin

REPORTABLE OUTCOMES

Meeting Abstracts for Poster Presentations

Thiel DA and Andrew Morris. *Characterization of the Lysophosphatidic Acid-producing Enzyme, Autotaxin.*
FASEB Summer Conference - Lysophosphatidic Acid

Thiel DA and Andrew Morris. *Regulation of Autotaxin/Lysophospholipase D by Intracellular Proteolysis.* 38th Annual Southeastern Regional Lipid Conference (Fall 2003).

CONCLUSIONS

The work summarized in this report presents observations that collectively provide the foundation for conducting further studies to characterize the enzyme activities of ATX as well as the biological effects of this molecule and the bioactive products of its enzyme activity, LPA and S1P, on breast cancer cells. Unchecked exposure of breast cancer cells to a relevant growth and survival factor, such as LPA, could be an advantage for the tumor as a whole. Insight into the mechanism of action for this particular enzyme including its trafficking, proteolysis, enzymatic activity and biological effects on cells would be an important contribution in breast cancer research because it would identify a new target for intervention in the disease.

I regret to report that I will no longer be pursuing my PhD and will be leaving my graduate program. The support from this predoctoral fellowship will be terminated as of August 17, 2004.

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