Extracellular Thimet Oligopeptidase is Carried by Cell Membrane Microvesicles of Human Prostate Cancer Cells

Yu Liu
ylli2@wellesley.edu

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Extracellular Thimet Oligopeptidase is Carried by Cell Membrane Microvesicles of Human Prostate Cancer Cells

Yu Liu

Submitted in Partial Fulfillment of the Prerequisite for Honors in Biological Chemistry

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ABSTRACT

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By Yu Liu

Peptidases often generate bioactive peptides that regulate and mediate intercellular communication. Although processing of peptide precursors is initiated intracellularly, additional modifications by peptidases are often completed extracellularly. Plasma membrane microvesicles (MVs or microparticles) carry membrane-associated molecules, including proteins, when released from their parental cells. Thimet oligopeptidase (TOP) is a biologically significant peptidase that is known to be secreted to the extracellular space. Since TOP has been localized on the external face of the cell plasma membrane, we asked whether TOP might be carried by cellular MVs into the extracellular space, and whether this process is influenced by steroid hormones. DU145 androgen-sensitive prostate cancer cells were treated with dihydrotestosterone (DHT). The cell culture supernatant was subjected to ultracentrifugation to pellet MVs. Western blot analysis of the homogenized MV sample revealed that TOP protein is indeed carried by the cellular MVs of both DHT-treated and control cells. MVs isolated from calcium ionophore-treated PC3 androgen-sensitive prostate cancer cells also carry TOP. Furthermore, activity assay results showed that the MV-associated TOP exhibits robust enzymatic activity. Thus, our studies strongly suggest that, in addition to the soluble form, extracellular TOP is also carried by cellular MVs. This is a novel form of the extracellular peptidase that may play important pathophysiological roles.
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**NOMENCLATURE**

A23187    calcium ionophore  
AR         androgen sensitive  
AtT-20     mouse LAF1 pituitary gland tumour cell line  
Ca²⁺       calcium ion  
DHT        dihydrotestosterone  
DMSO       dimethyl sulfoxide  
DNA        deoxyribonucleic acid  
DU145      human prostate cancer cell line  
ECL        western blotting detection kit  
FBS        fetal bovine serum  
FITC       fluorescein isothiocyanate  
GnRH       gonadotropin-releasing hormone  
GnRH-I     gonadotropin-releasing hormone analogue  
GPCR       G protein-coupled receptor  
HPG axis   hypothalamic-pituitary-gonadal axis  
kD         kilodalton  
LHRH       luteinizing-hormone-releasing hormone  
LNCaP      human prostate tumor cell line  
MCA        Methyl Coumarinyl Acetate  
MV, MP     microvesicle, microparticle  
NGS        normal goat serum  
PBS        phosphate buffered saline  
PC3        human prostate cancer cell line  
PMSF       phenylmethylsulfonyl fluoride, protease inhibitor  
PS         phosphotidylserine  
RNA        ribonucleic acid  
RPMI-1640  cell culture medium (Roswell Park Memorial Institute)  
TBS        Tris-buffered saline  
TCEP       Tris (2-carboxyethyl) phosphine hydrochloride  
TOP, E.C.3.4.24.15 thimet oligopeptidase
1. INTRODUCTION

1.1 Membrane Microvesicles

Intercellular signaling plays an important role in the progression and angiogenesis of tumor proliferation in cancers. It has long been understood that this intercellular correspondence depends upon the secretion and horizontal transfer of information between cells, specifically mediated through interactions between soluble ligands and their respective cell-associated receptors. However, recent discoveries highlight the existence of an alternative mediator of intercellular interactions. Microvesicles (MV, or microparticles) are small cellular membrane blebs which are released from the plasma membrane surface of almost all cell types upon cell activation or apoptosis (Fig. 1; Al-Nedawi et al., 2009; Dashevsky et al., 2009; Gyorgy et al., 2011a; Li et al., 2011, 2012; Rak, 2010). Research shows that microvesicles carry a vast array of molecular information such as a variety of biologically active proteins, oncogenic receptors and pieces of RNA material (Al-Nedawi et al., 2009). The formation, composition, general functions, catabolism and measurement of MVs have been the subject of recent studies and review articles (Freyssinet and Toti, 2010; Gyorgy et al., 2011b; Mause and Weber, 2010; Morel et al., 2011; Pisetsky and Lipsky, 2010; Rautou et al., 2011).
Microvesicles were first described as “platelet dust” over four decades ago, in studies describing the coagulation of platelet-rich plasma during storage {Wolf, 1967 #7066}. Typically, cellular MVs range from 100 nm to 1,000 nm in diameter (Gyorgy et al., 2011a). While microvesicles are often mistakenly grouped together with exosomes due to certain superficial similarities, they actually represent an entirely distinct population of structures. While exosomes originate from preformed intracellular vesicle formations that are released from the cell upon fusion from the plasma membrane, microvesicles are formed from outward budding of and shedding from the plasma membrane (Muralidharan-Chari et al., 2010). Despite several decades of intensive study, the exact mechanisms of the cellular vesiculation process remain unclear (Cocucci et al., 2009). The budding process consists of three key steps: sorting of protein cargo, budding of a portion of the plasma membrane (Muralidharan-Chari et al., 2009), and a loss of

Figure 1. The formation and release of microvesicles (MVs) upon cell activation and apoptosis. Image courtesy of Beyer, C. & Pisetsky, D. S. (2009).
standard membrane asymmetry resulting in the exteriorization of phosphatidylserine (PS) to the outer leaflet. Under standard physiological conditions, the lipid composition of the plasma membrane bilayer is asymmetrically distributed. While the internal leaflet is rich in aminophospholipids (phosphatidylserine (PS) and phosphatidylethanolamine), the external leaflet predominantly consists of phosphatidylcholine and sphingomyelin (Hugel et al., 2005). This membrane asymmetry is maintained by three key enzymes: aminophospholipid translocase, an inward-directed flippase pump specific for PS and phosphatidylethanolamine; a corresponding outward-directing “floppase”; and scramblase, which governs nonspecific bidirectional rearrangement of lipids across the membrane bilayer (Bevers et al., 1999; Hugel et al., 2005). Under certain cell activating conditions, such as a sustained influx of Ca2+ into the cytosol, membrane asymmetry may be lost as a result of the stimulation of scramblase and floppase activity coinciding with the inhibition of flippase (Fig. 2). These events are followed by the exposure of the internally facing PS to the external milieu and the subsequent release of microvesicles due to Ca2+ mediated proteolytic degradation of the cellular cytoskeleton. Due to the nature of their formation, MV molecular markers include phosphatidylserine externalization, Annexin V binding and tissue factor and cell-specific markers (Rak, 2010).
Given the nature of their formation, membrane MVs can harbor an assortment of different types of cargo when released from their parental cells, such as nucleic acids (DNA, RNA, microRNA) and membrane-associated lipids and proteins. The specific cellular cargo is dependent upon the specific cellular source and pathway of generation, and microvesicles

Figure 2. Plasma membrane remodelling and cellular microvesiculation in response to cell activation. Upon stimulation of the cell, a redistribution of the asymmetric bilayer occurs, leading to phosphatidylserine externalization and the outward budding and sectioning of microvesicles. Image courtesy of Hugel (2005).
studied under different experimental conditions have varied notably in their molecular and morphological makeup. Microvesicle shedding represents a fundamental process that is likely shared by virtually all cell types, healthy and diseased. As the microvesicular entity has been found to have considerable longevity once shed from source cell, all of its molecular cargo may be transferred horizontally between cells (Rak, 2010). In fact, studies have shown that cellular microvesicles play important roles as vessels for signaling mediation in a number of physiological and pathological pathways. Presently known key functions of MVs include: contribution to the pathogenesis of rheumatoid arthritis (Boilard et al., 2010; Distler et al., 2005; Jungel et al., 2007) and contribution to tumor metastasis (Giusti et al., 2008).

Routine methods for the isolation and analysis of microvesicles include differential centrifugation (Yuana et al., 2009), flow cytometry and capture based assays (Gyorgy et al., 2011a; Leroyer et al., 2010). Certain chemical agents, such as calcium ionophore (A23187), have also been used to induce the release of cellular microvesicles (Zhu and Wang, 1999). Physiologically, calcium is important for maintaining the potential difference across cell membranes and the differential calcium levels both inside and outside of the cell are tightly regulated, with intracellular calcium levels being over 100-fold lower than the external calcium concentration. At the onset of cellular apoptosis or under certain activating conditions, the level of intracellular calcium increases dramatically, which induces the release of cellular microvesicles (Kahner et al., 2008). Calcium ionophore (A23187) is known to increase the permeability of the plasma membrane to calcium, leading to rapidly increased calcium influx into the cell. Since the intracellular increase of calcium is closely associated with cell activation
and apoptotic events, calcium ionophore has been commonly used as a stimulus for studying cell membrane microvesicles (Dachary-Prigent et al., 1993).

1.2 An Introduction to Thimet Oligopeptidase

As previously discussed, the horizontal transfer of molecular components, such as peptide signals, represents a key aspect of intercellular communication. Bioactive peptide signals serve as important chemical messengers to mediate intercellular communication in multicellular organisms by binding to target cell surface receptors. Most peptides are initially synthesized as inactive precursors that require further proteolytic processing by peptidase enzymes in order to gain full functionality (Shrimpton et al., 2002). Thus peptidases play a critical role in the physiological generation and regulation of these bioactive peptide signals. Thimet oligopeptidase (TOP, E.C. 3.4.24.15) is a well characterized, 77 kD zinc metalloendopeptidase enzyme that hydrolyzes a number of physiologically important peptides in neuroendocrine pathways (Shrimpton et al., 2002). TOP is known to only hydrolyze short peptides of less than 20 residues in length, and recognize greatly variable cleavage sequences depending on the specific substrate in question (Checler et al., 1995; Checler et al., 1993; Dahms and Mentlein, 1992; Mentlein and Dahms, 1994). Although intracellular expression of TOP is known to be modulated at the level of transcription (McCool and Pierotti, 1998, 2000), its activity can also be regulated through posttranslational covalent modification (Shrimpton et al., 2002). TOP oligomerization through intermolecular disulfide linkage under non-reducing conditions result in a decrease or loss of
enzyme activity (Shrimpton et al., 1997; Shrimpton et al., 2003; Sigman et al., 2003). Recent studies report that the proteinous component of the cerebrospinal fluid impacts the oligomerization of TOP, which potentially represents another means for regulating secreted, extracellular TOP (Shrimpton et al., 2003).

TOP is known to be expressed in a wide range of mammalian tissues and cell types, such as the brain, pituitary gland and the testis (Acker et al., 1987; Chu and Orlowski, 1985; Moody et al., 1998; Pierotti et al., 1990; Pineau et al., 1999; Shrimpton et al., 2002; Swanson et al., 2004). Established physiological substrates of TOP include a number of important bioactive peptides of the central and peripheral nervous system, such as gonadotropin-releasing hormone (GnRH, also known as luteinizing-hormone-releasing hormone, LHRH), neurotensin, bradykinin, somatostatin, opioids, and angiotensin I (Barrett and Brown, 1990; Chu and Orlowski, 1985; Dahms and Mentlein, 1992; Dando et al., 1993; Mentlein and Dahms, 1994; Orlowski et al., 1983; Orlowski et al., 1989; Yang et al., 1994). Traditionally TOP has been categorized as a metabolizing enzyme that terminates neuropeptide function through hydrolysis, rendering these substrates unable to bind to their corresponding receptors (Dahms and Mentlein, 1992; Montiel et al., 1997). However new studies reveal that TOP also plays a number of alternative roles in the physiological metabolism of neuropeptides (Kim et al., 2003). First, it has been shown to transform inert precursor neuropeptides into their physiologically active forms (Acker et al., 1987). Second, it can convert one peptide into a different bioactive peptide which then either binds to a new receptor or initiates different downstream messages upon binding to the same receptor (Bourguignon et al., 1994). Finally, studies have shown TOP as a biomodulating enzyme, whereby a peptide product will act in opposition to its original parent peptide (Chappell
et al., 2004). Given its many roles in neuropeptide metabolism, TOP might also play a role in cell regulation. In fact, studies done using rat brain tissue show that TOP and its relevant peptide substrates are regulators of the G protein-coupled receptor (GPCR) signal transduction pathway (Cunha et al., 2008).

As noted, although a major portion of peptide processing may be initiated intracellularly, many peptides are further modified and activated in the extracellular milieu by enzymes such as TOP (Shrimpton et al., 2002). Thus TOP must be localized extracellularly as well as intracellularly, either released to the extracellular space as a soluble protein or associated with the plasma membrane on the cell surface. TOP has been found to be distributed in different subcellular locations depending on cell type, including nuclear localization, (Fontenele-Neto et al., 2001; Massarelli et al., 1999), cytosolic and secreted forms (Acker et al., 1987; Chu and Orlowski, 1985; Ferro et al., 1999; Fontenele-Neto et al., 2001; Garrido et al., 1999; Massarelli et al., 1999; Orlowski et al., 1983; Pierotti et al., 1990) and membrane association (Acker et al., 1987; Chu and Orlowski, 1985; Crack et al., 1999). In fact, studies have shown that TOP is secreted to the extracellular surroundings (Ferro et al., 1999; Garrido et al., 1999; Shrimpton et al., 2002) and TOP activity has been detected in the media of DHT-treated LNCaP androgen-responsive cells (Swanson et al., 2004). Furthermore, TOP has been visualized on the extracellular surface of the plasma membrane by confocal microscopy (Fig. 3; Crack et al., 1999).
1.3 Cellular Microvesicles, TOP and Cancer Progression

Intercellular communication is crucial to the progression and angiogenesis of tumor proliferation in cancers. As noted above, microvesicles serve as an alternative mediator of intercellular interactions and have been found to harbour a variety of biologically active proteins, oncogenic receptors and pieces of RNA material (Fig. 4; Al-Nedawi et al., 2009). Cellular
membranous microvesicles, which are called oncosomes in the field of cancer research, have been implicated in rapid intercellular transfer of oncogenic information between cells (Al-Nedawi et al., 2008; Di Vizio et al., 2009; Di Vizio et al., 2012). Furthermore, microvesicles obtained from the blood of cancer patients reveal the presence of oncogenes containing tumors and can thus potentially be used in a clinical setting as tell-tale prognostic and predictive biomarkers (Al-Nedawi et al., 2009).

Figure 4. Cellular microvesicles as mediators of intercellular communication. Image modified from figures courtesy of Mause et al, 2010 and Boon et al, 2013.
The roles played by a number of TOP-specific substrates in the proliferation of cancer have also become increasingly well documented. One area of particular interest is the role of TOP substrate GnRH and its analogues in the clinical management of prostate cancer. Early stage prostate cancer proliferation is dependent on male sex hormones (androgens) and can be effectively treated with androgen-ablation therapy. In fact, analogs of TOP substrate GnRH-I have been utilized in the treatment of prostate cancer since the early 1980s (Labrie et al., 1982; Plosker and Brogden, 1994), for which they are used to suppress the hypothalamic-pituitary-gonadal (HPG) axis during the early androgen dependent phase of prostate cancer. Recent studies have shown that GnRH in fact decreases prostate cancer cell growth (Labrie et al, 2005). While early stages of the disease have been effectively treated with androgen ablation therapy, later and more lethal stages of prostate cancer are often characterized by a progressive shift towards androgen independence, upon which time the cancerous cells no longer require androgen to proliferate and thus become less sensitive to traditional ablation therapy. In this more advanced phase, GnRH-I agonists appear to have a direct anti-tumor effect (Gnanapragasam et al., 2005).

Based on the aforementioned features of both the TOP enzyme and cellular MVs, we hypothesized that TOP may be carried by membrane MVs in the extracellular space under certain conditions. Yet online literature searches (via PubMed and Google) yielded no existing publications regarding the enzyme TOP and cellular MVs. It appears then, that whether MVs carry TOP in the extracellular space is still an unanswered novel question. The work described in this thesis include detection of TOP activity in the cell culture supernatant of both androgen-sensitive and insensitive DU145 and androgen sensitive PC-3 human prostate cancer cell lines, as well as in the cell-derived MVs. Additionally, we were able to isolate cellular MVs from the
cell culture supernatant and detect top protein in these MVs. We were also able to use calcium ionophore as a means of inducing increased release of microvesicle from the androgen sensitive PC-3 cells. Having obtained these promising preliminary data, we verified that the top carried by these cellular MVs is biologically active. In the future, it may also be interesting to further understand the manner by which calcium ionophore treatment stimulates microvesicle production and release. Since the MV-associated form of TOP has indeed been found to be biologically active, our results raise many interesting questions regarding whether this novel MV-carried form of TOP contributes to the proliferation of cancer proliferation.
2. MATERIALS & METHODS

2.1 Cell culture and treatment

The PC3-AR or DU145-AR cell lines (both androgen sensitive) were cultured at 37°C and at 5% CO₂ and grown in RPMI-1640 cell culture media consisting of 10% Fetal Bovine Serum (FBS) and pH 7.8. The cell medium was changed approximately every two to three days. The sub-confluent (~90%) DU145-AR cells, grown in T50 or T150 flasks, were treated without (control, ethyl alcohol) or with 0.1, 0.2 or 0.3 μM DHT for 24 hours (for the initial set of experiments) and with just 0.3 μM DHT for 24 hours (for the second set of experiments). Likewise, PC3-AR cells were treated without (control) or with 5 μM, 10 μM and 20 μM calcium ionophore (A23187) for 24 hours. After treatment incubation period, the cells were harvested and lysed in order to extract TOP. The supernatant was harvested for TOP activity assays and the purification of cellular microvesicles.

2.2 Method for Cell Lysis

Cells were harvested and washed in 1X Phosphate Buffered Saline (PBS) solution. Cell pellets were immersed in 12 times its volume of Kinetic Assay Buffer (25mM TRIS Hydrochloric acid, 125mM KCl, 1μM zinc chloride, 10% glycerol, pH 7.8 and conductivity 12.0mS/cm²). Phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, dissolved in isopropanol was freshly added in a ratio of 1:100 to the total volume of buffer. Cell pellets were kept in 70% ethyl alcohol ice slurry throughout the sonication process to prevent protein denaturation. Settings for sonication apparatus were: duty cycle set at 15 and the output control
set to 1.5. Individual pulse duration was one second and each pellet was pulsed 10-20 times, depending on pellet size. Sonicated cell samples were then centrifuged at 13,000 rpm and 4°C for 30 minutes. The supernatant was then harvested and stored at -80°C.

2.3 Concentration of cell culture supernatant

In order to detect TOP activity, the supernatants were harvested and concentrated using Amicon Ultra-4 centrifugal filter units with 10 kD Ultracel-10 membranes (Millipore) according to the manufacturer’s instructions.

2.4 Purification of cellular microvesicles from cell culture supernatant

To remove any residual cells, the supernatants from control and DHT-treated DU145-AR cells were centrifuged two times, each time at 3000 rpm for 15 min. The supernatant (~90%) was transferred into new centrifuge tubes after each spin, taking care not to disturb any cell pellets at the bottom of the tubes. Next, the supernatant samples were transferred to ultracentrifuge tubes (50 Ti rotor, Beckman Coulter) and ultracentrifuged at 33,000 rpm (100,000 g) for 30 minutes (Gyorgy et al., 2011; Li et al., 2011, 2012). The MV pellets from DHT-treated DU145-AR cells (Fig. 5) were washed by re-suspending in PBS and then merged into one ultracentrifuge tube, while the MV pellet from control cells was resuspended in PBS and stored for further study. The resuspended MVs of the combined DHT-treated samples were then ultra-centrifuged once again at 33000 rpm (1,000,000 g) for 30 minutes. The final MV pellets were re-suspended in 100μl of PBS with 2% SDS to use for subsequent experiments.
2.5 Assesment of Total Protein Concentration

Spectrophotometric analysis was used to determine the total protein concentration per sample. Absorbance was set at 260 nm and 280 nm for the spectrophotometer. Each cell lysate sample, obtained from sonication, was tested at a concentration of 1:25 in kinetic assay buffer.
Concentration of total protein for each sample was then calculated using the following formula:

\[
\text{Protein Concentration (mg/ml)} = (1.55 \times A_{280}) - (0.76 \times A_{260}).
\]

### 2.6 Western blotting for TOP protein in MV suspensions

Cell lysates were loaded to gels with equal amounts of protein per lane (10 μg/lane), while the homogenates of the isolated MVs were loaded at the maximum lane volume since our first goal was to detect whether the MVs carry TOP protein. Samples were prepared by combining the appropriate volume of each cell lysate sample (calculated based on total protein concentration), 5 μl of 4X SDS (Invitrogen) and 2 μl of 10X sample reducing agent (Invitrogen), then brought up to a final volume of 20 μl with water. Samples were then heated at 70°C for 5 minutes. Proteins were separated by size using NuPAGE Novex Bis-Tris gels (Invitrogen) and run at 200 V for ~35 minutes according to the manufacturer’s instructions. Separated proteins were then transferred onto a PVDF Immobilon-P membrane (Millipore), with voltage settings of 100 V for 1.5 hours. Next, the transferred membrane was blocked for 1 hour in 5% Carnation dry milk to prevent non-specific antibody binding, followed by overnight immuno-probing at 4°C with anti-rabbit IgG primary antibody (anti-TOP) at a concentration of 1:300,000 and anti-mouse IgG primary antibody (anti-actin) at a final concentration of 1:250,000. Primary antibodies were provided by Marc Glucksman (Rosalind Franklin School of Medicine). Secondary anti-TOP and anti-actin antibodies (GE Healthcare UK limited) were both used at final concentrations of 1:10,000 and were incubated for 1 hour at room temperature. The immune-probed membrane was then fluorescently labelled using an ECL developing kit for 5 minutes and then visualized using the Storm Scanner apparatus. Band intensity was quantified using ImageQuant software.
2.7 Immunocytochemistry

Cells were washed with 0.05M TBS (Tris-buffered saline) (pH 7.6). The cells were blocked in 20% normal goat serum (NGS) and 1% BSA in 0.05M TBS, then incubated in the primary antibody to TOP, monoclonal Rabbit-anti-TOP at 1:5000 in with 1% NGS in 0.05M TBS with 0.02% sodium azide, 0.1% gelatin (pH 7.6) for 24 hours. The cells were washed with 0.05M TBS with 0.02% sodium azide, 0.1% gelatin, and 10% Triton-X, then incubated in the secondary Donkey-anti-Rabbit Red 594 (Invitrogen cat# A21207) at 1:100 with 1.5% NGS in 0.05M TBS with 0.02% sodium azide and 0.1% gelatin for 90 minutes, then washed with the same buffer. The cells were incubated in nuclear stain DAPI at 1:10^6 with 1.5% NGS in 0.05M TBS with 0.02% sodium azide and 0.1% gelatin for 30 minutes, then washed with 0.05M TBS with 0.02% sodium azide and 0.1% gelatin, then washed with 0.05 TBS. The slides were coverslipped with Gel/Mount (biomeda, cat# M01).

**Imaging:** The cells were imaged using a Leica TCS SP5 II confocal microscope with the 40x oil lens at zoom = 2.00. Gain and offset were kept constant for each series of cells.

2.8 Assessment of TOP Activity with Quenched Fluorescence Assay using microplate reader

Cell lysates, cell culture supernatant, and purified MV suspensions were used for TOP activity assessment. 25mM TRIS HCl buffer (0.125mM KCl, 1μM ZnCl_2, 1mM TCEP, 10% glycerol) was used as the assay buffer. 0.1μM solution of wild type TOP was also prepared and used to ensure proper assay functioning. The artificial TOP substrate, Methyl Coumarinyl
Acetate (MCA, 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-Lys-dinitrophenol), was dissolved in DMSO and used at a concentration of 1.4 mM. The competitive inhibitor of TOP, N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-p-aminobenzoate (cFP) was dissolved in DMSO and was used at a concentration of 16.5 μM. cFP was added so as to assess breakdown that is not due to TOP activity in prostate cancer cells.

All fluorescence assays were conducted using a Molecular Devices SpectraMax M3 microplate reader. The emission wavelength was set at 400nm and the excitation wavelength was set at 325nm. The excitation width was fixed at 5nm, and 10nm emission slit was used. MCA (1 μl) and varying volumes of sample were brought to a final volume of 200 μl with assay buffer. The assays for cell lysates were carried out in duplicates when sample quantity permitted. Activity for each sample was monitored over 30 minutes, with a reading taken every minute. The slope of the graph (fluorescence intensity/minute) was then used to determine the activity of TOP. When needed, the activity from samples was subtracted from the blank, which consisted of 1ul of MCA and 199ul of assay buffer. Enzyme rate was then determined by calculating the slope (change in fluorescence per minute).
3. RESULTS

3.1 Detection of TOP activity in the supernatant of cultured DU145-AR cells

In the initial set of experiments, we sought to determine whether TOP activity can be detected in the supernatant of cultured DU145-AR cells without (control) or with 0.1 μM, 0.2 μM or 0.3 μM DHT treatment for 24 hours. Studies have reported that TOP activity could be detected in the media of the DHT-treated LNCaP androgen-sensitive prostate cell line (Swanson et al., 2004). Concentrating the supernatant by approximately 200-fold produced detectable TOP activity via the quenched fluorescence assay, thus confirmed that TOP activity does indeed exist in the extracellular milieu of DU145-AR cells. The results are in line with previous observation by Swanson et al (2004). Although TOP activity was clearly detected in the supernatants of both control and DHT-treated samples, the activity assay should be replicated with a larger sample size before any further conclusions can be drawn.

3.2 Observation of MV pellets from the supernatant of cultured DU145-AR cells

Having detected TOP activity with the concentrated supernatant, we next sought to isolate MVs from the cell culture supernatant. Untreated and DHT-treated cell culture supernatant samples were initially subjected to regular centrifugation to remove any detached cells and debris, followed with ultracentrifugation for MV pellet isolation. After ultracentrifugation, small opaque pellets were observed for untreated and DHT-treated samples. In order to maximize sample size, the MVs from five 0.3 μM DHT-treated flasks were combined
and re-ultracentrifuged to generate one single DHT-treated MV pellet. The untreated MV pellet measured ~1mm in width, while the DHT-treated pellet was ~3mm width-wise.

3.3 Detection of TOP protein in MVs isolated from the supernatant of cultured DU145-AR cells

In order to assess whether TOP is associated with cellular MVs, we used immunoblotting to probe for the presence of TOP proteins in cells and in MVs. Since TOP molecules carried by MVs originated from their parental cells, we began by probing for TOP protein in the cell lysates of cultured DU145-AR cells treated without (control) or with 0.3 μM DHT. TOP expression was clearly detected in both control and DHT-treated DU-145 cells (Fig. 6).

Having detected TOP expression in the cells, we next sought to determine whether cellular MVs derived from these cells carry TOP protein. MVs isolated from the supernatants of five DHT-treated DU145-AR cell flasks were combined to maximized sample size. The MV

Figure 6. Western blot analysis of TOP expression in cultured DU145 androgen-sensitive cells. Cell lysate samples for cells untreated (control) and treated with 0.3 μM DHT were analyzed with immunoblotting. TOP protein and loading control (actin) bands are shown.

Having detected TOP expression in the cells, we next sought to determine whether cellular MVs derived from these cells carry TOP protein. MVs isolated from the supernatants of five DHT-treated DU145-AR cell flasks were combined to maximized sample size. The MV
samples from both untreated and DHT-treated cells were homogenized with 0.2% TritonX-100, then analyzed via western blotting. The results indicated that cellular MVs from both treated and untreated cell cultures supernatants do indeed carry TOP protein, as indicated by the clearly visible TOP immunoblot bands (Fig. 7). The fact that the TOP enzyme is carried by cellular MVs in the extracellular space helped us to confirm our initial hypothesis and suggested that at least a portion of extracellular TOP exists with MVs, and not simply in the soluble format. Unlike for the cell lysates, total TOP protein concentration cannot be normalized to actin since it is clear that the amount of actin on MVs is not constant under all conditions, as it would need to be in order to serve as a proper loading control (Fig. 7). This is not surprising since MVs bleb off of the plasma membrane at random locations, meaning that the molecules associated with each MV would likely be different. Thus an alternative means of normalizing TOP protein concentration for MV samples is required.

Figure 7. Western blot analysis of TOP protein in MVs isolated from the supernatant of the cultured DU145 androgen sensitive cells. Cellular MVs isolated from untreated (control) and 0.3μM DHT-treated cell culture supernatants were analyzed via western blotting. In order to maximize sample size, DHT-treated MV sample lane was combined from MVs generated from approximately 2.5 flasks of supernatants.
3.4 Induction of Cellular Microvesicle Release from PC3-AR cells using Calcium Ionophore

From the above studies, we had determined that TOP is carried by cellular microvesicles from both DHT-treated and control DU145-AR cells. However, additional experiments with other cell types were necessary to confirm these novel findings. In addition, we had also dedicated much work to optimizing the isolation of microvesicles. However one remaining challenge was the small sample size of microvesicles obtainable from each experiment, which presented a limitation to the number and efficiency by which each experiment could be completed. It has been reported that the regulated release of microvesicles is induced upon subsequent increase of intracellular calcium concentrations (Kahner et al., 2008). Physiologically, calcium is important for maintaining the potential difference across cell membranes and the differential calcium levels both inside and outside of the cell are tightly regulated. Calcium ionophore (A23187) is known to increase the permeability of the plasma membrane to calcium, leading to rapidly increased calcium influx into the cell and has been commonly used as a stimulus for studying cell membrane microvesicles (Dachary-Prigent et al., 1993). Thus calcium ionophore was selected in order to stimulate the production and release of cellular microvesicles.

A dose study was conducted in order to determine the optimal concentration of calcium ionophore to be used as an induction agent of microvesicle release in future experiments. PC3-AR androgen sensitive human prostate cancer cells treated with 5 μM, 10 μM, 20 μM and untreated (control) for a period of 24 hours at 37°C. It should be noted that after the incubation
period, a clear dose-dependent increase in cell apoptosis was noted with increasing concentrations of calcium ionophore (Figure 8).

![Control PC3-AR](image1)
![5 μM A23187 treated PC3-AR](image2)
![10 μM A23187 treated PC3-AR](image3)
![20 μM A23187 treated PC3-AR](image4)

**Figure 8.** Concentration-dependent calcium ionophore treatment of PC3 androgen sensitive cells. Cells were incubated in 5 μM, 10 μM, 20 μM and untreated (control) at 37°C for an incubation period of 24 hours. Non-apoptotic cells after incubation are shown for each treatment group.

Treatment with 5 μM ionophore a minimal amount of apoptosis (~10%) and was comparable to the control. Compared to the control, the 10 μM ionophore treatment lead to moderate apoptosis (~40%) while 20 μM treatment caused near-uniform apoptosis (~90%). In addition to the cellular apoptosis that was observed, a dose dependent increase in the size of microvesicle pellets generated was also noted with increasing concentrations of calcium ionophore. These results
were not surprising considering the mode of action of calcium ionophore, which disrupts the transmembrane calcium concentration gradient and induces apoptosis, a prime condition for cellular microvesicle production and release.

Cell and microvesicle samples were harvested for western analysis. As expected, TOP was present in both calcium ionophore-treated and control cells (Fig. 9). Results also indicated that cellular microvesicles released from both ionophore-treated and untreated cells carry TOP protein, as indicated by visible TOP immunoblot bands.

Figure 9. Effect of calcium ionophore (A23187) concentration on th TOP expression in PC3 androgen sensitive cells and corresponding cellular microvesicles (MVs). Cells were treated with 5 μM and 20 μM lysates or untreated (control). Isolated cellular MVs were analyzed via Western blotting.

The amount of TOP detected on the cellular microvesicles of the 5 μM A23187 treatment group was comparable to that of the untreated group. As compared to the control group, the 20 μM calcium ionophore treated microvesicles had a notably clear band in the western blot (Fig. 9), which correlates to a greater amount of TOP protein. Subsequent experiments using treatment with 10 μM A23187 also showed an increase in the amount of TOP detected in cellular
microvesicles via western analysis (not shown). This observation is consistent with the dose-dependent increase in cellular microvesicle pellet size when treated with higher concentrations of A23187 as noted above. Furthermore, this suggests that treatment with A23187 may also increase the amount of TOP that is carried on cellular microvesicles. From the results of repeated dose studies, it should be noted that 10 μM A23187 was selected as the treatment concentration to be used for subsequent experiments. This choice was based on both the significant positive induction of microvesicle production and release observed in cells treated at this dose and the moderate amount of cellular apoptosis observed (~40%), which is significantly less than ~90% apoptosis for the 20 μM treated cells.

3.5 Assessment of relative amounts of intracellular, soluble extracellular and MV-carried TOP via fractionation

In order to determine the relative amounts of TOP that is localized intracellularly and extracellularly (freely soluble and carried by MVs), repeated fractionation studies were conducted using PC3 androgen sensitive prostate cancer cells. Cells were untreated (control) and treated with 10 μM calcium ionophore. The cell lysate containing intracellular TOP, concentrated pre-ultracentrifuged supernatant (containing both soluble and MV TOP), concentrated post-ultracentrifuged supernatant (soluble TOP only) and purified MV samples (MV-associated TOP) were analyzed in parallel using Western blotting. Our very preliminary results indicate that ~5% of the total amount of TOP produced by the cell is carried on microvesicles in 10μM A23187-treated cells. However, additional repeat experiments are
required in order to obtain more precise quantitative data regarding the relative distribution of TOP in the separate fractions. Results for the supernatant fractions were inconclusive due to remaining challenges that exist with concentrating and handling the supernatant. Specifically due to the highly viscous nature of the concentrated supernatant fractions (>40X concentrated), these samples could not be properly separated using gel electrophoresis. The study was also repeated using less concentrated (25X) supernatant samples. However TOP concentration studies were too low to be clearly detected by the antibody. Thus it appears that further optimizations must be made to the supernatant concentration protocol and additional repeat studies will be required. An enzyme-linked immunosorbent assay (ELISA) could also be considered as an alternative means of assessing the relative amounts of TOP in each of the fractions (Thompson A et al. 1997). This could provide a high-throughput alternative for assessing TOP levels in liquid samples that would also be very sample efficient.

3.6 Immunocytochemical Imaging of Cellular Microvesicles and Microvesicle-Associated Thimet Oligopeptidase with Confocal Fluorescent Microscopy

In order to document the display of TOP on the surface of A23187-treated and untreated human prostate cancer cells, we used immunocytochemistry and fluorescent confocal microscopy. PC3 androgen sensitive cells were treated either with 10 μM A23187 or without
(control) for 24 hours before cells were fixed. Figure 6 shows control and A23187 treated cells stained with TOP specific primary antibody followed by anti-Rabbit Red 594 (red) to detect TOP and FITC-conjugated Annexin V (green) to detect phosphotidylserine exposure.

The orange/yellow colored blebs in the merged images demonstrates co-localization of the TOP and externalized phosphotidylserine. The presented confocal images clearly show a concentration of cell-surface TOP in small, circumscribed domains rich in externalized phosphotidylserine, a marker of cellular microvesicle generation. The sizes of these domains are consistent with the accepted size range for microvesicles (approximately 0.1 to 1 μm) and

Figure 10. Confocal fluorescent images demonstrating the display of cell-surface TOP of PC3 androgen sensitive cells. Cells were either treated with 10 μM A23187 or untreated (control), as indicated, for 24 hrs. Cells were then chemically fixed and detected with anti-TOP antibody (red, stained with anti-Rabbit Red 594) and FITC-labeled Annexin V (green, which preferentially binds externalized phosphatidylserine). Confocal slices were taken to visualize the upper surface of the cells. The yellow color in the merged images (Merge) demonstrates co-localization of the two labels (indicated by arrows). Scale bar 1 μm.
resemble published images of microvesicle release (Huber et al., 2002; Liu et al., 2012). Thus, A23187 treatment appears to preferentially localize TOP into plasma membrane blebs, presumably as part of the export of this molecule onto MVs. Furthermore, the images show a concentration of cell-surface TOP as a result of induction of cell-surface TOP on A23187-treated cells compared to the control cells, which had minimal amount of detectable TOP on their surface (Fig. 10). These results confirm and extend our findings with western analysis of whole-cell homogenates as presented previously (Fig. 7, 9).

![Figure 11. Confocal fluorescent images demonstrating the heterogeneity of cell-surface TOP of PC3 androgen sensitive cells.](image)

It is important to note that cellular microvesicles are characteristically defined by their heterogeneity and the non-uniform array of molecular cargo that they carry, a feature that is
largely due to the nature of their formation and release from the parent cell surface. This heterogeneous nature is clearly demonstrated in Figure 11, which shows a large concentration of cell-surface TOP as a result of massive induction of cell-surface TOP on a cell undergoing apoptosis that was treated with 10 μM A23187. Again the small, circumscribed, cell-surface domains that stained intensely for both TOP and externalized phosphotidylserine exhibited sizes in the range for microvesicles. The presented image clearly depicts an example where these cell surface, circumscribed TOP rich domains are present in much greater quantities than typically observed in the average cell population (Fig. 10). Furthermore as seen in the merged image, the already released microvesicles surrounding the cell display different amounts of co-localization with cell surface TOP. Although representing a rather extreme case of cell-surface TOP localization, this image helps illustrate the heterogeneity of cellular microvesicles and the array of molecular cargo they may carry.

3.7 Assessment of TOP activity in cellular microvesicle suspensions isolated from the cell culture supernatant of PC3-AR cells

To determine whether the TOP carried by cellular microvesicles is enzymatically active, we carried out quenched fluorescence activity assays. Cells were treated with 10 μM A23187 or untreated (control) for 24 hrs and then the cells and the cell culture supernatant were then collected for preparation of cell lysate and microvesicle suspensions, respectively. In both
treated and control sets, the amount of total protein added from the cell lysate was equivalent to 1/20 the amount added for the microvesicle suspensions.

![Figure 12. Assessment of TOP activity in cells and cellular microvesicles. Cells were treated with 10 μM A23187 for 24 hrs. Enzymatic activity of TOP was determined using quenched fluorescence kinetic assay, by the cleaving of fluorescent-tagged artificial substrate (7-methoxycoumarin-4-yl)acetate (MCA). Enzyme rates, as depicted above, were obtained by calculating the slopes (change in fluorescence per minute).](image)

Enzyme rates were calculated in terms of change in fluorescence per unit time. Results from the activity assay are shown in Figure 12. Not surprisingly, robust TOP activity was detected in both
control and A23187-treated cell lysate samples, which confirms existing knowledge that enzymatically active TOP is present in PC3 androgen sensitive cells.

Interestingly, robust TOP activity was also detected in the microvesicle suspensions of both control and A23187-treated samples. Furthermore, both sets of microvesicle suspension samples exhibited dose-dependent effects, whereby incrementally increasing the amount of sample (and hence increasing the amount of TOP protein) added to the assay lead to incrementally greater enzyme rates (Fig. 12). These findings again confirm the presence of TOP on cellular microvesicles and also clearly demonstrate that this microvesicle-associated form of extracellular TOP is enzymatically active. TOP enzyme rates measured from the microvesicles released from A23187-treated cells were significantly higher than the enzyme rates of the corresponding control microvesicle group. The opposite effect was seen for the cell lysates, whereby the control group exhibited a significantly higher level of TOP activity compared to the treatment group. Together these observations appear to be consistent with the mode of action of the ionophore treatment in inducing cellular apoptosis and microvesicle generation. This also suggests that treatment with A23187 promotes intracellular TOP to be localized to the extracellular face of the plasma membrane, at which point the active enzyme is carried away from the cell by microvesicles into the extracellular milieu.
4. DISCUSSION

In the current literature, TOP is thought to be a soluble molecule in the extracellular milieu. However, our study demonstrates for the first time that TOP is also released into the extracellular space as a form that carried on microvesicles when released by apoptotic human prostate cancer cells into the extracellular space (Fig. 10, 11). Most importantly, we showed that the TOP carried on microvesicles in the extracellular space exhibits robust enzymatic activity (Fig. 12). In the current study, we have identified that the microvesicle-carried TOP can be released from two different prostate cancer cell lines; MV-associated TOP is not only released from calcium ionophore induced apoptotic PC3 prostate cancer cells (Fig. 9), but also from untreated DU145 prostate cancer cells (Fig. 7). The latter results suggest that microvesicle carried TOP may also exist under natural conditions without additional stimulation.

As previously discussed, many physiological substrates of TOP, such as GnRH, bradykinin and neurotensin, are found either in the extracellular space or in the blood. Thus in order to fully modulate its physiological substrates, TOP must be accessible to the extracellular space, either by exposure on the surface of the cell plasma membrane or release from the cell as a soluble, free protein. Previous studies have localized TOP on the extracellular surface of the plasma membrane (Crack et al., 1999) and have shown that TOP is secreted out to the extracellular surroundings as a soluble free protein (Ferro et al., 1999; Garrido et al., 1999; Shrimpton et al., 2002). Thus this format of microvesicle-associated TOP likely represents a novel extracellular form of the enzyme in addition to the soluble form.
Studies have shown that microvesicles released by cancer cells appear to play an important role in tumor progression and therapy resistance. For example, MVs released by ovarian cancer cells that carry the Fas ligand appears to contribute to tumor cell escape from immune detection by causing T-cell apoptosis (Inal et al., 2012; Pap et al., 2011). The role played by a number of TOP-specific substrates in the proliferation of cancer cells is a topic of interest in the field of cancer research. Early stage prostate cancer proliferation is dependent on male sex hormones (androgens) and can be effectively treated with androgen-ablation therapy. Analogs of TOP substrate GnRH-I have been utilized in the treatment of prostate cancer since the early 1980s (Labrie et al., 1982; Plosker and Brogden, 1994), for which they are used to suppress the hypothalamic-pituitary-gonadal (HPG) axis during the early androgen dependent phase of prostate cancer. Recent studies have shown that GnRH in fact decreases prostate cancer cell growth (Labrie et al, 2005).

As a key modulator of the activities of GnRH and its other physiological substrates, extracellular TOP stands as an important subject of study in further understanding the pathophysiological significance of these molecules. The current study demonstrated a novel format of extracellular TOP as being carried by microvesicles when released from prostate cancer cells. In contrast to the soluble format of TOP, which easily diffuses into the circulation and can be diluted by the large volumes of the blood stream, the microvesicle carried TOP may stay in the tumor microenvironment in a relatively high concentration. This microvesicle-associated format of TOP may enable the enzyme to work more potently on its substrates, therefore contributing to the progression of pathological conditions. Thus, further understanding of the activities and functions of MV-associated TOP in the extracellular space and the
mechanisms of how TOP-bound and released cell membrane microvesicles pose a very interesting area of study in developing more effective therapeutics for the treatment of prostate cancer and related pathologies.
5. REFERENCES


