MolecularMedicine

Regulation of Autophagy by α_1-Antitrypsin: "A Foe of a Foe Is a Friend"

Michal G Shapira,[1] Boris Khalfin,[1,2,3] Eli C Lewis,[2] Abraham H Parola,[1,3] and Ilana Nathan[2,4]

[1]Department of Chemistry, Faculty of Natural Sciences, and [2]Department of Clinical Biochemistry and Pharmacology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel; [3]New York University Shanghai, People's Republic of China; and [4]Soroka University Medical Center, Beer-Sheva, Israel

Autophagy is involved in both the cell protective and the cell death process but its mechanism is largely unknown. The present work unravels a novel intracellular mechanism by which the serpin α_1-antitrypsin (AAT) acts as a novel negative regulator of autophagic cell death. For the first time, the role of intracellularly synthesized AAT, other than in liver cells, is demonstrated. Autophagic cell death was induced by *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and tamoxifen. By utilizing a fluorescently tagged TPCK analog, AAT was "fished out" (pulled out) as a TPCK intracellular protein target. The interaction was further verified by competition binding experiments. Both inducers caused downregulation of AAT expression associated with activation of trypsin-like proteases. Furthermore, silencing AAT by siRNA induced autophagic cell death. Moreover, AAT administration to cultured cells prevented autophagic cell death. This new mechanism could have implications in the treatment of diseases by the regulation of AAT levels in which autophagy has a detrimental function. Furthermore, the results imply that the high synthesis of endogenous AAT by cancer cells could provide a novel resistance mechanism of cancer against autophagic cell death.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2014.00054

INTRODUCTION

Autophagy is involved in both cell protective and destructive processes (1,2) in various diseases, such as cancer, neurodegenerative diseases, pathogen invasion and other muscular and liver disorders (3–5). A key clinical issue is to devise therapeutic approaches to regulate autophagy either by inhibition of the autophagic process when harmful, for example, in pancreatic cancer where autophagy induces resistance to treatment, or by its beneficial activation during starvation or chemotherapy.

Most proteases known to be involved in autophagy are cysteine proteinases like autophagins (6) and calpain (7). Scant information, however, is available, pertaining to a putative role of serine proteases and their inhibitors serpins in the modulation of the autophagic cell program. It has been reported that a serine protease gene, *isp6*$^+$, is involved in autophagy in *Schizosaccharomycespombe* (8), and serine protease inhibitor Kazal type 3 (*Spink3*) null mice exhibit autophagic cell death of pancreatic cancer cells (9). Furthermore, a recent study has been shown that *Omi* (also known as *HtrA2*), a heat-shock–induced serine protease, is a positive regulator of autophagy. *Omi* activates autophagy through digestion of *Hax-1*, a *Bcl-2* family–related protein that represses autophagy in a Beclin-1 (mammalian homologue of yeast *Atg6*)–dependent pathway (10).

The 52-kDa α_1-antitrypsin (AAT) is a member of the serpin family. AAT, a major blood protein, inhibits neutrophil elastase, proteinase-3, cathepsin G, thrombin, trypsin and other proteinases (11). Of note, AAT was shown to protect islet cells from apoptotic death (12).

The present study reports the novel finding that, in addition to the well-recognized extracellular AAT activity, this serine protease inhibitor also modulates autophagy from within the cell.

The chymotrypsin-like protease inhibitor *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) has been

With respect to the motto in the title, see The Oxford Dictionary of Proverbs*, 5th edition: "The enemy of my enemy is my friend."*

Address correspondence to *Abraham H Parola, Ben-Gurion University of the Negev, Beer-Sheva, Israel. Phone: +972-8-6472454; +972-52-8795945; Fax: +972-8-6472943; +86-138-1629-3057; E-mail: aparola@bgu.ac.il; ap137@nyu.edu; or to Ilana Nathan, Ben-Gurion University of the Negev, Beer-Sheva, Israel. Phone: +972-7-6400263; +972-58-423-65-61 (cell); Fax: +972-7-6281361; E-mail: nathan@exchange.bgu.ac.il.*

Submitted March 21, 2014; Accepted for publication July 29, 2014; Epub (www.molmed.org) ahead of print July 30, 2014.

used in a number of studies to investigate signal transduction pathways that are involved in gene expression, cell proliferation and regulation of cell death. In addition to its binding to chymotrypsin, TPCK interacts with other cell proteins (13). While in a number of studies TPCK appears to protect cells against cell death (14–17), a proapoptotic effect of TPCK was reported in several other cell models (18,19).

Utilizing a fluorescently tagged TPCK analog, we "fished out" (pulled out) AAT as a TPCK intracellular protein target. We show in the present study for the first time that TPCK induces autophagic cell death by downregulation of intracellular AAT. These results provide a new cellular mechanism regulating autophagic cell death by binding and downregulating the levels of the intracellular synthesized serpin, whose cellular role was hitherto unknown. Cumulatively, these findings and our observation that extracellular AAT also functions as a negative regulator of autophagy may afford new therapeutic strategies for intervention against diseases in which prevention of autophagy is warranted. Since AAT is already clinically used and is well tolerated even at high concentrations, its way to additional clinical applications will be relatively short.

MATERIALS AND METHODS

Cell Culture and Treatments

The human breast cancer MCF-7 cell line was cultured in DMEM (Invitrogen [Thermo Fisher Scientific Inc., Waltham, MA, USA]) supplemented with 10% FBS (Biological Industries, Kibbutz Beit-Haemek, Israel), 300 mg/L L-glutamine (Biological Industries), 100 U/mL penicillin, 100 μg/mL streptomycin and 12.5 U/mL nystatin (Biological Industries) and 100 U/mL recombinant human insulin (Biological Industries), at 37°C in 5% CO_2.

HT-29 was cultured in RPMI 1640 (Invitrogen [Thermo Fisher Scientific Inc.]) supplemented with 10% FBS, 300 mg/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 12.5 U/mL nystatin at 37°C in 5% CO_2.

Cells were plated in 24-well plates or 96-well plates at a concentration 5×10^4 cells/mL. A day later, the cells were treated with different concentrations of TPCK (Sigma-Aldrich, St. Louis, MO, USA) and tamoxifen (Sigma-Aldrich) as indicated in the figures, for the indicated time with or without the inhibitor 3-MA (Sigma-Aldrich) or bafilomycin A1 (LC Laboratories, Woburn, MA, USA). Cell viability was determined by trypan blue staining.

Labeling of Autophagic Vacuoles with Monodansylcadaverine (MDC)

Following treatment, the cells were incubated with 0.05 mmol/L MDC (Sigma-Aldrich) in phosphate-buffered saline (PBS) at 37°C for 10 min (20). For visualization of MDC-labeled vacuoles, cells were grown on coverslips, incubated with MDC, washed four times with PBS and immediately analyzed by fluorescence microscopy.

Protein Lysates

Normal or treated 10^7 cells were collected, washed twice with ice-cold PBS and resuspended in 0.5 mL ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% sodium deoxycholate) for Western blotting or in lysing buffer (50 mmol/L Tris-HCl pH 7.5, 0.1% NP-40, 1 mmol/L dichlorodiphenyltrichloroethane [DTT], 0.25 mol/L sucrose, 2 mmol/L $MgCl_2$) for enzyme activity assays. The cells were broken by polytron cell homogenizer (4 cycles of 7 s) and the cell debris pelleted by centrifugation in an ultracentrifuge at 120,000*g* for 30 min, at 4°C. The supernatant was used immediately or stored at –70°C. Protein content of each sample was determined with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA) according to the Bradford method using bovine serum albumin (BSA) (Sigma-Aldrich) as standard.

Western Blot Analysis

Equal amounts of protein samples (40 μg/lane) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred to polyvinylidenedifluoride (PVDF) membranes. The membranes were blocked with 5% skim milk/Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h, after which the samples were incubated overnight at 4°C with each of the above primary antibodies: rabbit anti-LC3 (MBL, Woburn, MA, USA) or goat anti-AAT or sheep anti-actin (Santa Cruz Biotechnology, Dallas, TX, USA) polyclonal antibodies. The membranes were then washed three times with TBST, followed by 1 h incubation at room temperature with the secondary antibody an HRP-conjugated anti-rabbit/sheep IgG (Jackson ImmunoResearch, West Grove, PA, USA). The membranes were then washed three times with TBST and were visualized using an EZ-ECL Chemiluminescence Detection Kit for HRP (Biological Industries).

siRNA Transfection

For long-term transfection, MCF-7 cells were transfected with 0.02 μmol/L control negative siRNA or AAT siRNA (Qiagen, Hilden, Germany) using the HiPerFect Transfection reagent. When the cells become confluent (after 72 h), they were split and transfected again.

Morphological Quantification of Apoptosis

Cells undergoing morphological changes associated with apoptotic cell death were stained with 5 mg/mL acridine orange (Sigma-Aldrich) and 5 mg/mL ethidium bromide (Sigma-Aldrich) (AO/EB) and monitored by a fluorescence microscope as described by McGahon *et al*. (21). Cells were scored as alive if their nuclei exhibited normal morphology and were green. Cells exhibiting normal morphology and orange color were identified as necrotic, and if their nuclei exhibited condensation of the chromatin and/or nuclear fragmentation, they were identified as apoptotic.

Cell Cycle Analysis

Cell cycle phase distribution treatment was analyzed by flow cytometry using propidium iodide (PI) (Sigma-Aldrich) staining (11). Control and treated cells were harvested by trypsin, washed with PBS, then fixed in 95% ethanol and stored at 4°C for up to 7 d before DNA analysis. After the removal of ethanol by centrifugation, cells were incubated in a solution containing 10 μg/mL of RNase A (Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) at room temperature for 45 min. Cells were then stained with 15 μg/mL PI. Stained nuclei were analyzed for DNA-PI fluorescence using a Cytomics FC500 flow cytometer (Beckman Coulter Inc., Brea, CA, USA).

Annexin V–Fluorescein Isothiocyanate (FITC) Staining

Staining for annexin V-FITC binding was carried out on cells exposed to treatments for 24 h. Cells were washed in PBS and resuspended in the binding buffer (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, pH 7.4). The cell suspension was incubated with annexin V-FITC. After 15 min, cells were washed twice in binding buffer and were analyzed by flow cytometry.

Labeling Chymotrypsin-Like Proteases with Fluorescence Analogs of TPCK

FSFCK (carboxyfluorescein labeled) or Texas Red-L-phenylalanyl-chloromethyl ketone (TRFCK) are fluorochrome-labeled analogs of TPCK. The cells were treated with 10 μmol/L of FSFCK and TRFCK at different time intervals. Following treatment, the cells were washed three times with PBS and immediately analyzed by fluorescence microscopy or by flow cytometry.

Purification of TPCK-Bound Protein by Column Chromatography

TRFCK was incubated with control lysates or with 50 μmol/L TPCK-treated lysates for 30 min at 37°C. Solid ammonium sulfate (Sigma-Aldrich) was added to the lysates to 40% (w/v) saturation and kept at 4°C for 1 h through gentle stirring. The precipitate was collected by centrifugation at 12,000*g* for 30 min, followed by dissolution in 1:5 volume (v/v) 50 mmol/L Tris-HCl buffer, pH 7.5 (wash buffer). This fraction was desalted by dialyses with wash buffer and kept at 4°C overnight through gentle stirring. Then the sample was loaded to a DEAE-sepharose anion exchange column, which was previously equilibrated with wash buffer. Elution was done with 85 mL of a salt gradient of 0–0.5 mol/L NaCl in wash buffer at a flow rate of 1 mL/min absorbance at 280 nm and fluorescence of TRFCK at emission maximum (em): 615 nm and excitation maximum (ex): 596 nm of the fractions (each 1 mL). Fractions containing fluorescence were pooled, desalted and concentrated by ultrafiltration (10-kDa cutoff, Vivaspin; Vivaproducts, Inc. Littleton, MA, USA). Affinity chromatography was performed on anti–Texas Red sepharose beads, which were equilibrated previously with wash buffer. The samples were kept at 4°C overnight through gentle stirring. The bound protein was eluted from the beads using wash buffer plus 0.1 mol/L NaOH pH 10. Concentrated elutes were analyzed by SDS-PAGE and fluorescence band was identified by mass spectroscopy analysis.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

The extract was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) according to the method of Weber and Osborne (22). Equal amounts of protein samples (40 μg/lane) were subjected to 10% SDS-PAGE. After electrophoresis, the gel was stained with 0.2% Coomassie blue and protein bands were visualized after destaining in methanol:acetic acid:water (1:1:8).

Binding Experiment between Recombinant AAT and TRFCK

Two μg/μL AAT was incubated with or without 0.05 mmol/L tamoxifen for 30 min at 37°C, followed by incubation with 50 μmol/L TRFCK for 30 min at 37°C. Laemmli sample buffer (×5) was added to the samples and the samples were boiled at 95°C for 5 min. The samples were analyzed by Western blotting using goat anti-AAT polyclonal antibody. In addition, TRFCK fluorescence of the same membrane was detected by UV light.

Enzyme Activity Assays

Enzymatic assay of AAT inhibition of trypsin activity was determined according to Bergmeyer *et al*. (23). All assays were set up at a total volume of 1 mL by adding 1.625 μg/mL trypsin (Sigma-Aldrich) to 0.23 mmol/L *N*-α-benzoyl-L-arginine ethyl ester (Sigma-Aldrich) substrate in 63 mmol/L sodium phosphate in the presence of AAT and TPCK at various concentrations. The samples were immediately mixed by inversion and the increase in A_{253nm} was recorded for approximately 5 min in spectrophotometer and the maximum linear rate was calculated.

Trypsin activity in cell lysates was determined according to Warner *et al*. (24). All assays were set up at a total volume of 200 μL by adding 20 μg proteins, 0.5 mmol/L *N*α-benzoyl-L-arginine p-nitroanilide hydrochloride (Sigma-Aldrich) as substrate in the presence or absence of 50 μmol/L TLCK and set up to 200 μL with 50 mmol/L Tris-HCl pH 7.5. The samples were added in triplicate to a flat-bottomed 96-well plate. After incubation for 24 h at 37°C, the optical density (OD) of 405 nm was measured with a plate enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA). The protease activity was calculated according to the calibration curve of p-nitroanilide.

Electrophoresis on Fluorescence Substrate Gel

Electrophoresis on fluorescence substrate gel was performed as previously described (25). Proteases were reversibly inactivated by adding 40 μL protein lysate containing 80 μg protein to 10 μL 60 mmol/L Tris-HCl buffer, pH 6.8, with 25% glycerol, 2% SDS, 10% sucrose and 0.1% bromophenol blue. Samples were

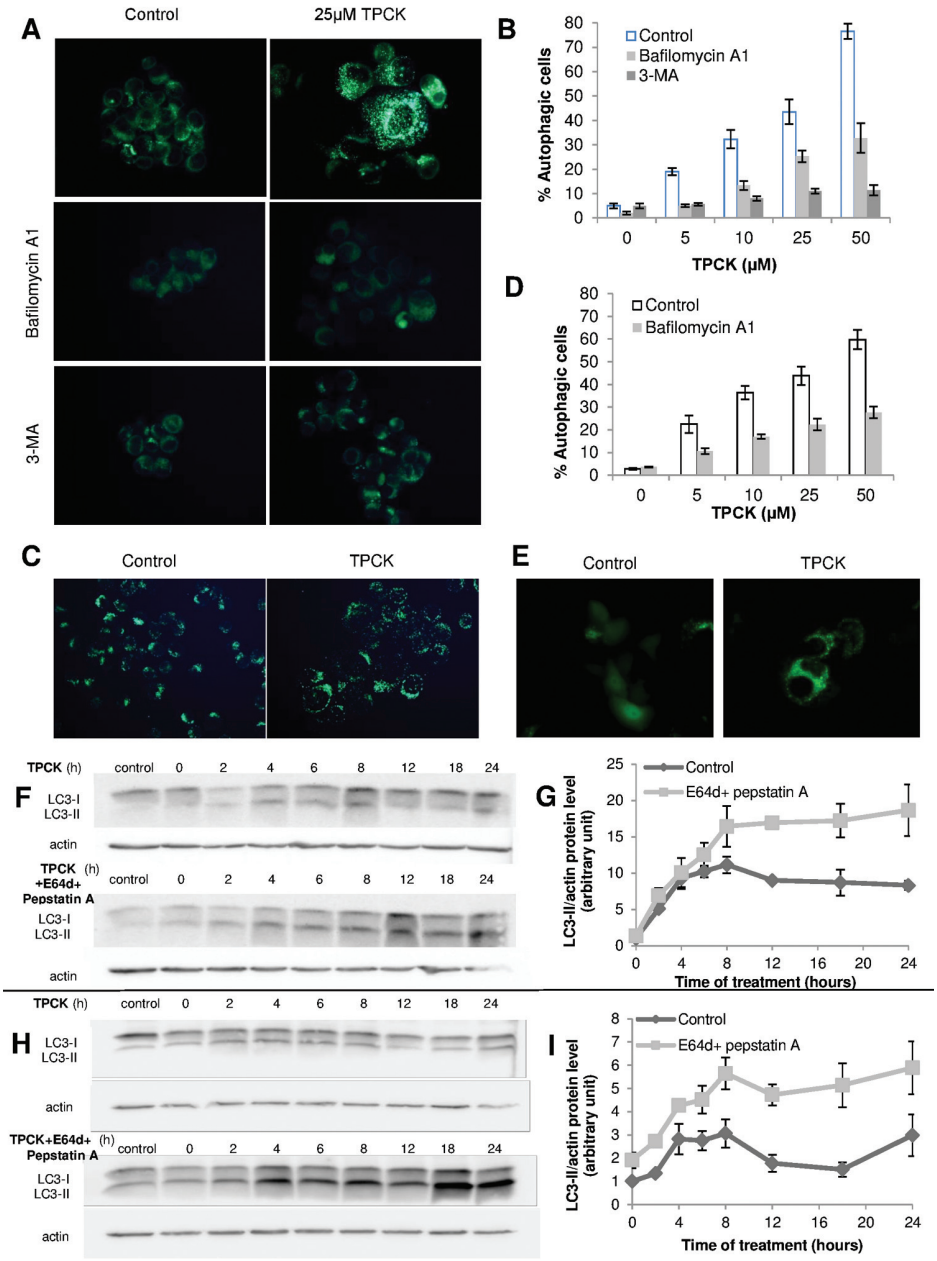

Figure 1. TPCK induces autophagy. TPCK-induced autophagy is evident by fluorescence microscopy: (A) MCF-7 and (C) HT-29 cells treated with TPCK, with or without 3MA (5 mmol/L) or bafilomycin A1 (50 nmol/L) for 24 h, and stained with MDC; magnification 40×. The quantification of the results obtained with (B) MCF-7 and (D) HT-29 cells, mean ± SE of at least four independent experiments. (E) Images of GFP-LC3 transfected MCF-7 cells treated with TPCK (50 μmol/L) for 24 h. (F,G) MCF-7 and (H, I) HT-29 cells treated with TPCK (50 μmol/L) for different times, and LC3-II levels were analyzed by Western blots. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$, compared with control, by Student *t* test.

then electrophorated using 200 μmol/L T-butyloxycabonyl-L-glutaminyl-L-alanyl-L-arginine 4-methylcoumaryl-7-amide (Sigma-Aldrich) copolymerized in a 10% polyacrylamide gel. After electrophoresis, the gels were subjected to three repeated immersions in 0.1 mol/L Tris-HCl buffer, pH 7.0, containing 2.5% (v/v) Triton-X100 to remove the SDS and reactivate the proteases. The gels were incubated for 1 h at 37°C in 0.1 mol/L glycine-NaOH buffer, pH 7.0. The bands of protease activity were detected under UV light.

Statistical Analysis

Each experiment was performed 3 to 5 times at least in duplicates. The results indicate mean ± SE. Statistical analysis was performed using a Student *t* test. Significance was set at $P < 0.05$.

RESULTS

TPCK Induces Autophagy in MCF-7 and HT-29 Cell Lines

In our search for the role of serine proteases in autophagy, we unexpectedly discovered that TPCK, a chymotrypsin-like protease inhibitor, induces autophagy in MCF-7 and HT-29 cells (Figure 1). Autophagy was confirmed by the auto fluorescent marker of autolysosomes, monodensyl cadaverin (MDC). MDC staining of TPCK-treated cells exhibited a dose-dependent increase in fluorescence intensity and in the number and size of MDC-labeled vesicles (Figures 1A–D). In addition, GFP-LC3 transfected MCF-7 cells treated with TPCK show accumulation of this label, indicating an increased number of autophagosomes (Figure 1E). Moreover, the autophagy inhibitors 3-methyladenine (3-MA) (which blocks autophagosome formation via the inhibition of type III phosphatidylinositol 3-kinases [PI-3K]) and bafilomycin A1 (a V-ATPase inhibitor which prevents maturation of autolysosomes) both suppressed MDC uptake in MCF-7 cells treated with TPCK (see Figures 1A, C, D). Furthermore, Western blots of TPCK-treated cells revealed the appearance of LC3-II (Figures 1F–I), one of the hallmarks of autophagy (26). In addition, coincubation with the lysosomal protease inhibitors E64d and pepstatin A (which blocks the last steps of autophagosome fusion with the lysosome and prevent protein degradation) enhanced TPCK-induced accumulation of LC3-II, confirming that TPCK induces dynamic autophagy in MCF-7 and HT-29 cells.

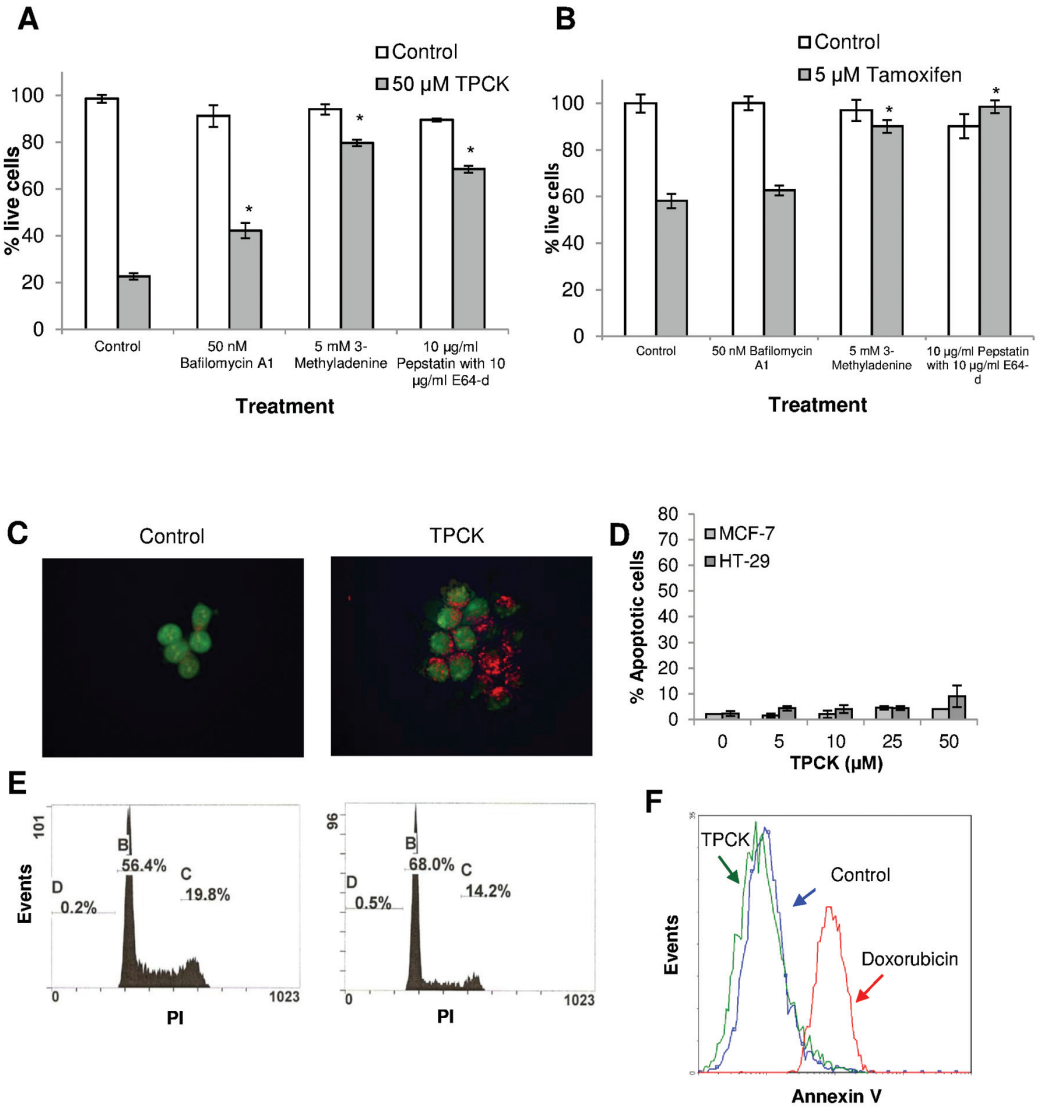

Figure 2. TPCK causes autophagic cell death and not apoptosis. Autophagy in MCF-7 cells was induced by treatment with (A) 50 µmol/L TPCK for 24 h or (B) 5 µmol/L tamoxifen for 72 h; 30 min before the autophagy induction the cells were treated with 50 nmol/L bafilomycin A1 or 5 mmol/L 3-methyladenine or with combination of 10 µg/mL pepstatin and 10 µg/mL E64-d. Cell viability was assessed by trypan blue staining (expressed by percentage of viability of DMSO-treated cells (n = 3)), *$P < 0.02$ as compared with DMSO-treated respective controls. (C) Fluorescence microscopy analysis of AO/EB staining, magnification 40×. (D) Quantification of AO/EB-stained cells treated with different concentrations of TPCK for 24 h. Flow cytometry analysis of (E) cell cycle and (F) annexin V-FITC of MCF-7 cells treated with control DMSO or TPCK (50 µmol/L) for 24 h.

TPCK and Tamoxifen Induce Autophagic Cell Death

Autophagy can be involved in both cell death and cell survival. Our results show that the effects of TPCK and tamoxifen in this system are related to autophagic cell death.

Thus, TPCK and tamoxifen induced cell death in MCF-7 cells, as revealed by trypan blue staining. In addition, inhibition of autophagy by 50 nmol/L bafilomycin A1, 5 mmol/L 3-methyladenine or by a combination of 10 µg/mL pepstatin with 10 µg/mL E64-d reduced TPCK- or tamoxifen-induced cell death (Figures 2A, B).

TPCK has been shown to induce apoptotic cell death in different cell lines. In addition, autophagy can be associated with apoptosis. Cell apoptosis was not detected by acridine orange/ethidium bromide double staining (Figures 2C, D). Notably, acridine orange granular accumulation of orange color in addition to the green nuclear staining was observed in TPCK-treated cells, indicative of activation of lysosomes and autophagy. Fur-

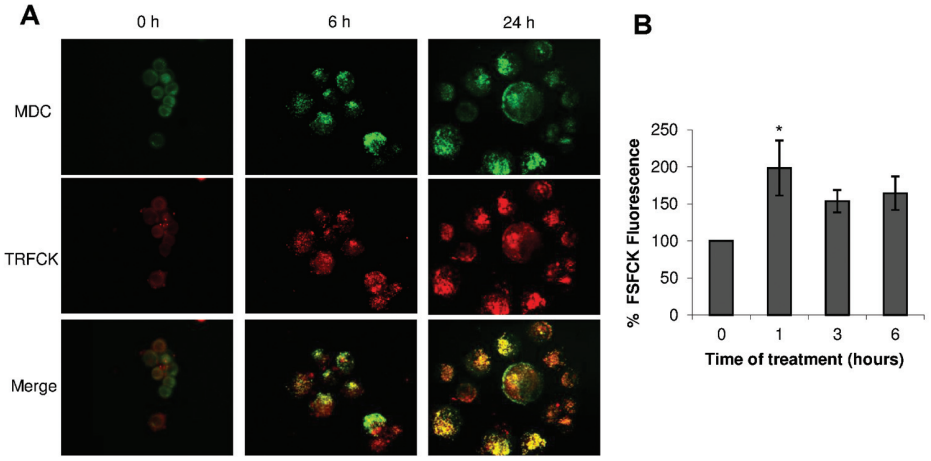

Figure 3. FLISP induces autophagy. TRFCK induces autophagy as seen by fluorescence microscopy of MDC-labeled and TRFCK-treated (10 μmol/L) MCF-7 cells (A). Flow cytometry analysis of FSFCK-treated (10 μmol/L) MCF-7 cells (B). *$P < 0.05$, compared with time 0, by Student *t* test.

thermore, TPCK-treated MCF-7 cells were negative for annexin V-FITC staining compared with doxorubicin-treated cells, which served as a positive control for apoptosis (Figure 2E).

In addition, we performed cell cycle analysis. TPCK treatment did not cause the appearance of the sub-G_0/G_1 cells, indicative of apoptosis, although it led to some decrease in frequency of DNA-replicating (S-phase) cells and a corresponding increase in the proportion of G_0/G_1 cells (Figure 2F).

To pull out the proteins with which TPCK interacts, we used fluorochrome labeled inhibitors of serine protease (FLISP), a fluorescent analog of TPCK (27). First, we had to ascertain that the tagged TPCK do indeed cause autophagy (Figure 3A). Next we found out that the FLISP-labeled proteins were not observed in the autolysosomes 6 h after treatment but reached it only at a later stage, 24 h after treatment. This implies a lack of involvement of the fluorescently tagged proteins in the direct formation of the autophagosome. However, it is presumably involved in autophagy induction.

Isolation of the TPCK-Bound Protein

To identify the TPCK target protein, we prepared protein lysate of control and TPCK-treated cells. TRFCK was added to both lysates and the fluorescently tagged protein was purified by ammonium sulfate precipitation (Figure 4A), followed by separation on DEAE-sepharose anion exchange column (Figures 4B, C) and on an affinity column to which an antibody to Texas Red is bound (Figure 4D). Most of the protein of control cells was precipitated after the addition of 40% ammonium as measured by the fluorescence of TRFCK. After running the protein on SDS-PAGE a major fluorescent band at about 50 kDa was evident (see Figure 4A).

Separation on DEAE-sepharose anion exchange column by a step gradient of NaCl yielded several protein peaks as measured by absorbance at 280 nm (see Figure 4B). The major fluorescence intensity of TRFCK was detected in fractions 53–61, indicating high labeling specificity which was not the case in fractions 68–78 in which the specific fluorescent labeling was much lower. The collected fractions were concentrated and loaded on SDS-PAGE. After staining with Coomassie blue, we got a major band in fractions 53–62 corresponding to a 50-kDa molecular weight (MW) (see Figure 4C). Figure 4D shows the results of purification of the collected 53–62 fractions by an affinity column to which an antibody to Texas Red is bound. To finally identify the TRFCK-reactive protein, we compared control with TPCK-treated cell lysates. As expected, we got a major 50-kDa fluorescence band with the control lysate, which was remarkably stronger than that observed with TPCK-treated lysate. This was expected since the control lysate should show a fluorescent band corresponding to the TRFCK–reactive protein, while TPCK-treated lysate should not, since there is no free target protein left. To identify the TRFCK-reactive protein, we extracted the protein from the gel after trypsin digestion and subjected it to mass spectrometry. Unexpectedly, the TPCK-bounded protein was identified as α_1-antitrypsin (AAT), which is a 52-kDa serine protease inhibitor that belongs to the serpin family. We further explored whether TPCK can bind to AAT and inhibit its activity *in vitro*. Recombinant AAT was mixed *in vitro* with TRFCK and the sample was separated by SDS-PAGE. A fluorescent band of TRFCK at about 50 kDa was observed (Figure 4E, lane 4), indicating covalent binding between the two. Moreover, when denatured AAT was mixed with TRFCK, no fluorescent band was observed (Figure 4E, lane 1), meaning that TRFCK binds only to the intact AAT protein. The identity of the fluorescent protein as AAT was validated by Western blotting with an antibody against AAT (Figure 4E). Next, we investigated if TPCK prevents AAT inhibition of trypsin activity in the cells, (Figure 4F). As expected, AAT inhibits trypsin activity. When TPCK was added, the inhibition by AAT was abolished, suggesting that TPCK binding to AAT reduces AAT inhibition of trypsin.

Next, the influence of TPCK and tamoxifen (a known inducer of autophagy) on AAT protein levels in MCF-7 cells and HT-29 was investigated. Western blot analysis of MCF-7 (Figures 5A, B) and HT-29 (Figures 5C, D) cell lysates reveals that AAT level decreased during TPCK and tamoxifen treatment as compared with actin. Since the half lifetime of AAT is relatively long, 3-4 d, the fast decrease in AAT levels following 1–3 h treatment suggests that the protein un-

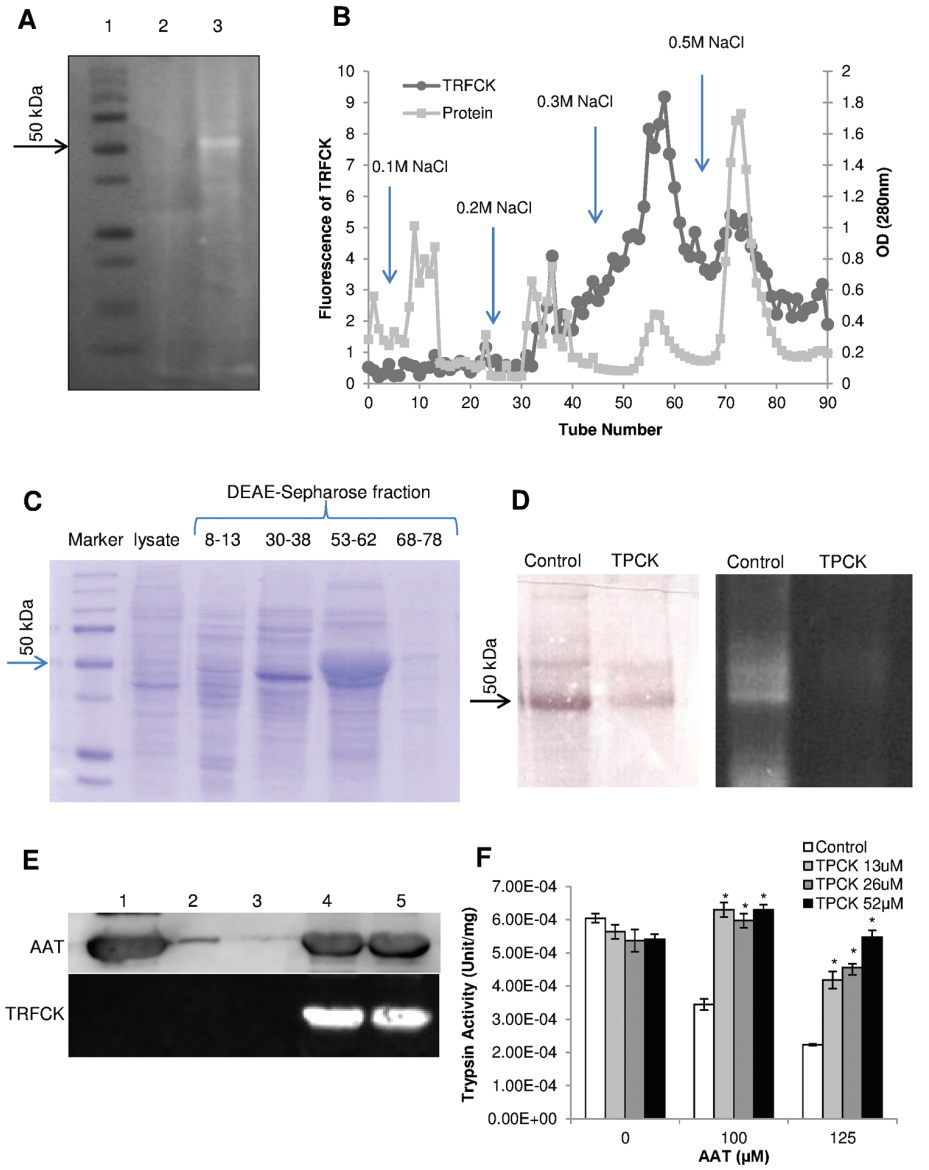

Figure 4. The fishing out of AAT. Lysates of control and TPCK-treated (50 μmol/L, 1 h) MCF-7 cells were incubated with TRFCK (50 μmol/L, 30 min) followed by purificating the fluorescently labeled protein. (A) UV analysis of SDS-PAGE of control lysate, precipitated with 40% ammonium sulfate: lane 1, marker; lane 2, lysate; lane 3, precipitated proteins. (B) Elution profile of DEAE-sepharose column of the precipitate of the control lysate. (C) Coomassie blue analysis of the concentrated fractions from DEAE-sepharose column of the control lysate. (D) Coomassie blue and UV analysis of control and TPCK-treated lysate after the final affinity column. TRFCK binds *in vitro* covalently to AAT as analyzed by immunoblot and UV; AAT (50 μg/mL) was incubated first with tamoxifen (0.5 mmol/L, 30 min) followed by incubation with TRFCK (50 μmol/L, 30 min): lane 1, denatured AAT + TRFCK; lane 2, TRFCK; lane 3, tamoxifen; lane 4, AAT + TRFCK; lane 5, AAT + tamoxifen + TRFCK (E). TPCK inhibits AAT inhibition of trypsin as measured by enzymatic assay (F). *$P < 0.001$, compared with control, by Student *t* test.

dergoes degradation. This suggests that during autophagic cell death, AAT presumably degrades. Since tamoxifen also causes a decrease in AAT level, we examined if tamoxifen also binds to AAT by competition-binding experiments using TRFCK. Tamoxifen did not cause a reduction in TRFCK fluorescence (Figure 4E, lane 5) suggesting that tamoxifen does not bind to the TRFCK-binding site on AAT and thus does not interfere (for example, by competition or steric hindrance) with the binding of AAT to TRFCK. In addition, we further investigated whether inhibition of AAT in the cells will induce autophagy. MCF-7 cells transfected with siRNA for AAT showed an increase in autophagy after 6 d as measured by Western blot for LC3-II (Figures 5E–F).

Would externally adding recombinant AAT to cells inhibit autophagic cell death? The addition of AAT to the cells prior to induction of autophagy by both TPCK and tamoxifen results in reduction in autophagy, assessed by MDC staining (Figure 5G, H) and in cell death, determined by trypan blue staining (Figure 5I). This suggests that recombinant AAT presumably competes with the native AAT on binding to TPCK and prevents autophagic cell death.

Antitrypsin is a potent inhibitor of trypsin- and elastase-like proteases. We thus examined whether downregulation of AAT levels during autophagy is associated with activation of these enzymes in the cells. Indeed, induction of autophagy by both inducers, TPCK and tamoxifen, caused an increase in intracellular trypsin-like activity measured by zymography using a fluorescent substrate (Figure 6A) or by a colorimetric enzymatic assay (Figure 6B). TPCK treatment caused the appearance of two bands, and the enhancement of two other bands, representing four different trypsin-like proteases. However, no change in elastase activity was evident (data not shown).

DISCUSSION

We have shown that AAT is a new intracellular regulator of autophagic cell death. We propose that, while AAT normally prevents autophagic cell demise by binding trypsin-like proteases, once the autophagic program is induced, AAT expression is downregulated leading "by default" to the unimpeded ac-

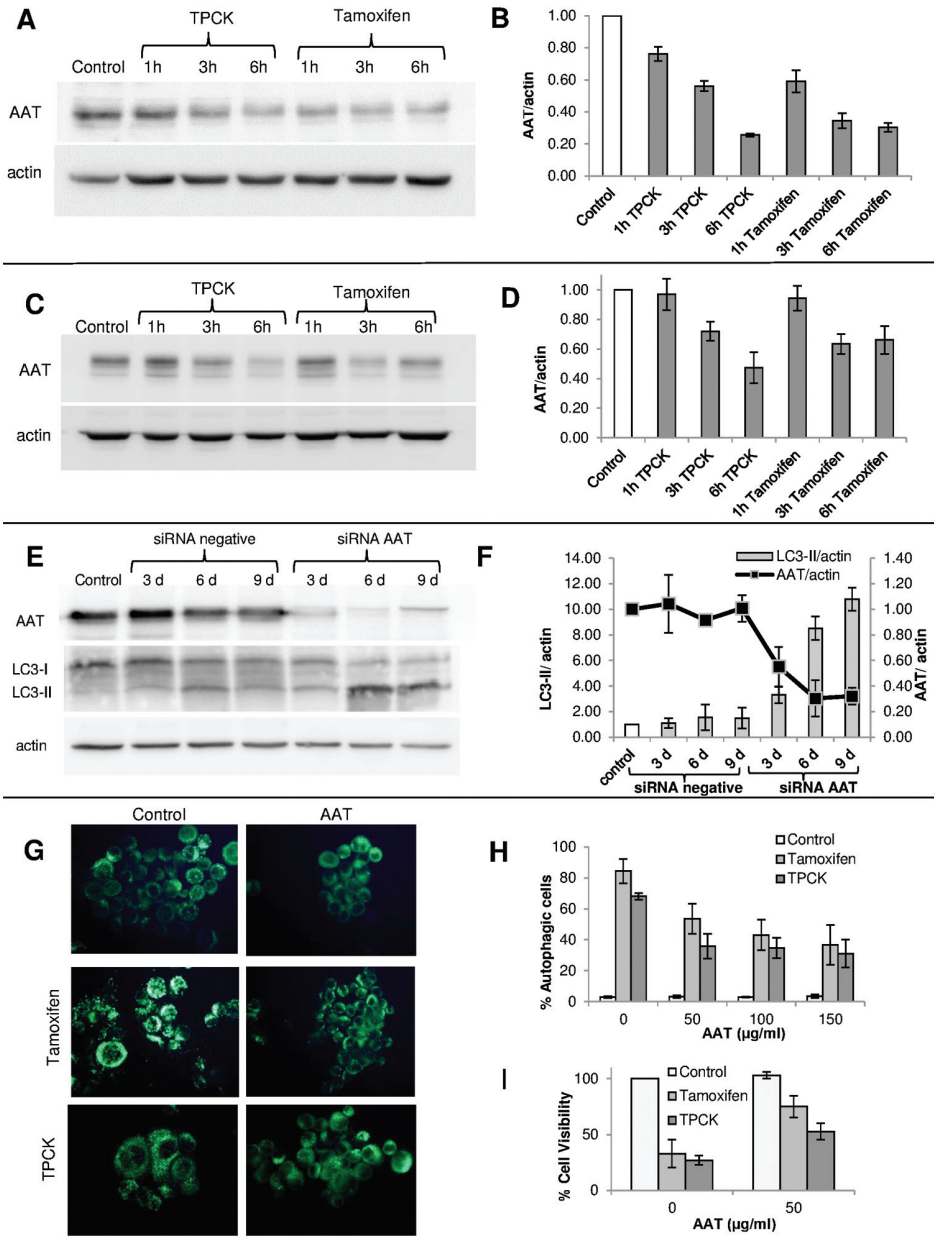

Figure 5. AAT modulates autophagy. AAT is downregulated by TPCK (50 μmol/L) and tamoxifen (10 μmol/L) in (A,B) MCF-7 and (C,D) HT-29 cells, detected by immunoblotting. AAT silencing causes autophagy induction. (E,F) MCF-7 cells were transfected with siRNAs, and LC3-II and AAT levels analyzed by immunoblotting. AAT inhibits autophagic cell death induced by TPCK (50 μmol/L, 24 h) or tamoxifen (5 μmol/L, 72 h) in MCF-7 cells as (G) seen and (F) analyzed by fluorescence microscopy and measured by trypan blue staining (I). magnification 40×. Data represent mean ± SE of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (versus control); $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ (versus without AAT), by the Student *t* test.

tivity of trypsin-like proteases ultimately promoting cell death by autophagy.

Our results are consistent with the observation that SPINK3-deficient mice exhibit autophagic death of pancreatic acinar cells (9). Thus, a novel mechanism, governed by an intracellular serine protease inhibitor appears to regulate autophagy in humans and rodents. While the role of serine proteases in autophagic cell death has not attracted much attention, our study indicates an involvement of both serine proteases and of antitrypsin in cell autophagy (Figure 6).

Earlier studies showed that while at a low concentration, TPCK prevented intranucleosomal cleavage of DNA (19), while at high concentrations, TPCK induced apoptotic death of cells of disparate lineages (18,28). To date, however, there is no information pertaining to a functional involvement of TPCK in the cell death mode of autophagy. The present results show that TPCK 5–50 μmol/L) administration to MCF-7 and HT-29 cells resulted in an increased uptake of MDC, a specific marker for autophagic vacuoles (20), in a concentration-dependent manner compared with control cells. Of note, the autophagy inhibitors, 3-MA and bafilomycin A1, both suppressed MDC uptake in MCF-7 cells treated with TPCK. Moreover, GFP-LC3-transfected cells treated with TPCK showed an accumulation of the MDC label, indicating an increased number of autophagosomes, and an increase in LC3-II in a time-dependent manner was noted by Western blot assay (Figure 1).

Activation of the autophagic program may result either in cell death or cell survival (1). We have demonstrated that TPCK induces autophagic cell death rather than survival, and that inhibition of autophagy by different chemical autophagy inhibitors reduced TPCK-induced cell death (Figure 2). Notwithstanding that TPCK was shown to cause apoptosis in several cell lines (18,19), cell death by apoptosis in MCF-7 and HT-29 cells was excluded by the following findings: (a) TPCK did not cause condensation of the chromatin or fragmentation of the DNA as assessed by AO/EB staining (Figure 2C); (b) early phosphatidyl serine exposure, as measured by annexin V-FITC, was not observed (Figure 2F) and; (c) TPCK treatment did not cause the appearance of pre-G_0/G_1 phase in cell cycle analysis indicative of apoptotic cells (Figure 2E).

An array of biological factors controls the switching between apoptosis and au-

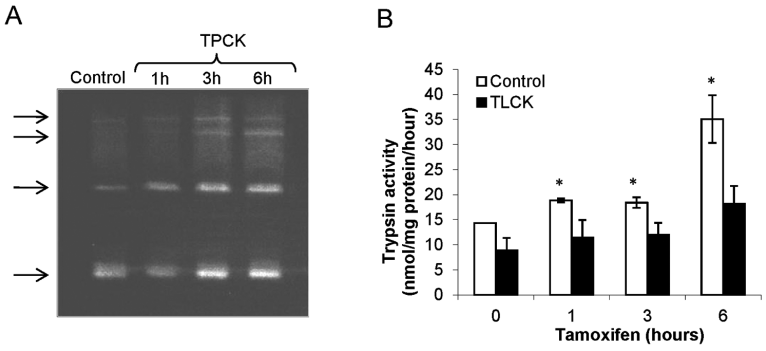

Figure 6. TPCK and tamoxifen induce trypsin-like activity. Zymography using fluorescent trypsin substrate of the lysate obtained from TPCK-treated MCF-7 cells (A). Time dependence of the colorimetric assay for trypsin-like protease activity of lysate obtained from tamoxifen-treated (10 μmol/L) MCF-7 cells with or without TLCK (50 μmol/L) in the assay (B). Data represent mean ± SE of at least three independent experiments. *$P < 0.05$, compared with time 0, by Student *t* test.

tophagy (29). Those include calpain-mediated cleavage of Atg5, which leads to inhibition of autophagy and promotes apoptosis (7). The mechanisms underpinning induction by a given compound of different modes of cell death in different cell lines remain to be clarified.

The use of fluorescent analogs of TPCK indicated that the TPCK-bound protein was not involved directly with autophagosomes formation since colocalization of TRFCK with MDC occurs at the late stage of the autophagic cell death (Figure 3).

We present evidence that AAT is a TPCK-target protein (Figures 3, 4). This interesting observation was further verified by *in vitro* studies using TRFCK aimed at assessing both the covalent binding of the TPCK analog and its ability to impede a key AAT biological function, such as the inhibition of trypsin activity. One may argue that a protease inhibitor suppresses the activity of another protease inhibitor, ultimately leading to autophagic cell death, a clear instance of the motto, "A foe of a foe is a friend." The TPCK binding to AAT could be associated with its antiprotease activity but not necessarily so since TPCK is known to interact with other nonproteolytic proteins (13). Of note, tamoxifen is not a protease inhibitor and its mechanism of action in downregulation of AAT activity remains to be clarified.

AAT is found mainly in plasma. This serpin is produced primarily by liver cells (70% to 80%), but it is also synthesized by monocytes, macrophages, pulmonary alveolar cells, and by intestinal and corneal epithelia (11). *De novo* synthesis of AAT also has been demonstrated in human cancer cells and strongly expressed AAT-positive cases have poor prognosis. The function of these high levels of endogenous AAT is not yet clear (30). The present study may suggest a novel resistance mechanism by which cancer cells protect themselves from undergoing autophagic cell death by synthesis of AAT.

Until recently, it was thought that the primary function of AAT is the inhibition of blood neutrophil elastase and proteinase 3. However, recent studies indicate that AAT is an irreversible inhibitor of other extracellular serine proteases, such as trypsin and kallikreins (5) and matriptase (31–33). Interestingly, AAT that was taken up by cells can inhibit apoptotic cell death via inhibition of caspase 3 (34). The present study shows for the first time that intracellular synthesized AAT is a regulator of autophagic cell death. Thus, a new pathway by which autophagy inducers could activate cell death by the interaction between serpin and AAT, changing the serpin–proteases balance and leading to serpin downregulation in the cells. This is further supported by the results showing activation of trypsin-like proteases.

In the past decade, clinical-grade affinity-purified AAT has resurfaced as a potential therapy for multiple clinical indications in which it is either inactivated (as in diabetes) or partially lacking (as in pregnancy complications), as well as conditions in which its cytoprotective attributes appear to be beneficial (ischemic heart disease) or its immunomodulatory attributes are much needed (steroid-resistant graft versus host disease) (35). There are currently nine clinical trials that examine AAT for nondeficient individuals: six for type 1 autoimmune diabetes, two for GvHD and one for IHD. In addition, trials examining its benefit for cystic fibrosis patients is ongoing, using an inhaled form of AAT. None of the trials was required to begin as a phase I study, as the safety is markedly evident from three decades of administrating AAT at high doses to individuals with AAT deficiency, once a week, for life (36) (superseding normal values throughout half of the intervals between infusions). The current trials are all either phase IIa or phase IIb. More trials are submitted as this manuscript is being evaluated, including the use of AAT for Crohn's disease based on very positive preclinical data.

The present study indicates that AAT is a versatile drug endowed with wide clinical applications, in addition to its use in AAT deficiency disease. Thus, AAT can be potentially used to prevent autophagy, for example in pancreatic cancer, in which autophagy is required for tumor growth or in Alzheimer's disease to impede impaired autophagy which plays a contributory role in the development of this devastating disease (37,38). On the other hand, the development of nontoxic AAT inhibitors or silencing the AAT gene could induce autophagic cell death in malignancies such as breast cancer in which mutational events impair cell death by apoptosis (39). Consistently, AAT was shown to interfere with tumor growth in an experimental system that focused on tumor-associated angiogenic processes (40); also,

AAT-deficient individuals also are treated with AAT exhibit a marked reduction in lung-cancer frequency. Our research may lead to a revolutionary breakthrough in treatment of apoptosis-resistant cancers. Moreover, this treatment may be more tolerable than currently used chemotherapy with its devastating side effects.

ACKNOWLEDGMENTS

We are grateful to Zbigniew Darzynkiewicz from the Brander Cancer Research Institute at the New York Medical College for providing us with FSFCK and TRFCK and for his giving advice. The financial support of the James-Frank Center for Laser-Matter Interaction, the Edmund Safra Foundation for Functional Biopolymers and the NYUSH research grant to AH Parola are gratefully acknowledged.

DISCLOSURES

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Codogno P, Meijer AJ. (2005) Autophagy and signaling: their role in cell survival and cell death. *Cell Death Differ*. 12 Suppl 2:1509–18.
2. Klionsky DJ, Emr SD. (2000) Autophagy as a regulated pathway of cellular degradation. *Science*. 290:1717–21.
3. Shintani T, Klionsky DJ. (2004) Autophagy in health and disease: a double-edged sword. *Science*. 306:990–5.
4. Levine B, Kroemer G. (2008) Autophagy in the pathogenesis of disease. *Cell*. 132:27–42.
5. Chen N, Karantza V. (2011) Autophagy as a therapeutic target in cancer. *Cancer Biol. Ther*. 11:157–68.
6. Marino G, *et al*. (2003) Human autophagins, a family of cysteine proteinases potentially implicated in cell degradation by autophagy. *J. Biol. Chem*. 278:3671–8.
7. Yousefi S, *et al*. (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat. Cell. Biol*. 8:1124–32.
8. Nakashima A, *et al*. (2006) A starvation-specific serine protease gene, isp6+, is involved in both autophagy and sexual development in Schizosaccharomyces pombe. *Curr. Genet*. 49:403–13.
9. Ohmuraya M, *et al*. (2005) Autophagic cell death of pancreatic acinar cells in serine protease inhibitor Kazal type 3-deficient mice. *Gastroenterology*. 129:696–705.
10. Li B, *et al*. (2010) Omi/HtrA2 is a positive regulator of autophagy that facilitates the degradation of mutant proteins involved in neurodegenerative diseases. *Cell Death Differ*. 17:1773–84.
11. Hallak M, *et al*. (2008) A molecular mechanism for mimosine-induced apoptosis involving oxidative stress and mitochondrial activation. *Apoptosis*. 13:147–55.
12. Zhang B, *et al*. (2007) Alpha1-antitrypsin protects beta-cells from apoptosis. *Diabetes*. 56:1316–23.
13. Ballif BA, Shimamura A, Pae E, Blenis J. (2001) Disruption of 3-phosphoinositide-dependent kinase 1 (PDK1) signaling by the anti-tumorigenic and anti-proliferative agent n-alpha-tosyl-l-phenylalanyl chloromethyl ketone. *J. Biol. Chem*. 276:12466–75.
14. Karahashi H, Nagata K, Ishii K, Amano F. (2000) A selective inhibitor of p38 MAP kinase, SB202190, induced apoptotic cell death of a lipopolysaccharide-treated macrophage-like cell line, J774.1. *Biochim. Biophys. Acta*. 18:207–23.
15. Lotem J, Sachs L. (1996) Differential suppression by protease inhibitors and cytokines of apoptosis induced by wild-type p53 and cytotoxic agents. *Proc. Natl. Acad. Sci. U. S. A*. 93:12507–12.
16. Eitel K, Wagenknecht B, Weller M. (1999) Inhibition of drug-induced DNA fragmentation, but not cell death, of glioma cells by non-caspase protease inhibitors. *Cancer Lett*. 142:11–6.
17. Huang Y, Sheikh MS, Fornace AJ Jr., Holbrook NJ. (1999) Serine protease inhibitor TPCK prevents Taxol-induced cell death and blocks c-Raf-1 and Bcl-2 phosphorylation in human breast carcinoma cells. *Oncogene*. 18:3431–9.
18. Heussler VT, Fernandez PC, Machado J Jr., Botteron C, Dobbelaere DA. (1999) N-acetylcysteine blocks apoptosis induced by N-alpha-tosyl-L-phenylalanine chloromethyl ketone in transformed T-cells. *Cell Death Differ*. 6:342–50.
19. Zhu H, Dinsdale D, Alnemri ES, Cohen GM. (1997) Apoptosis in human monocytic THP.1 cells involves several distinct targets of N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK). *Cell Death Differ*. 4:590–9.
20. Biederbick A, Kern HF, Elsasser HP. (1995) Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *Eur. J. Cell Biol*. 66:3–14.
21. McGahon AJ, *et al*. (1995) The end of the (cell) line: methods for the study of apoptosis in vitro. *Methods Cell Biol*. 46:153–85.
22. Weber K, Osborn M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem*. 244:4406–12.
23 Bergmeyer HU (ed.). (1974) *Methods of Enzymatic Analysis*. 2nd English edition. New York: Academic Press. 4 vols.
24. Lavens SE, Proud D, Warner JA. (1993) A sensitive colorimetric assay for the release of tryptase from human lung mast cells in vitro. *J. Immunol. Methods*. 166:93–102.
25. Yasothornsrikul S, Hook VY. (2000) Detection of proteolytic activity by fluorescent zymogram in-gel assays. *Biotechniques*. 28:1166–8, 70, 72–3.
26. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E. (2005) Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy*. 1:84–91.
27. Grabarek J, Darzynkiewicz Z. (2002) *In situ* activation of caspases and serine proteases during apoptosis detected by affinity labeling their enzyme active centers with fluorochrome-tagged inhibitors. *Exp. Hematol*. 30:982–9.
28. Wu M, *et al*. (1996) Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells. *EMBO J*. 15:4682–90.
29. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat. Rev. Mol. Cell. Biol*. 8:741–52.
30. Higashiyama M, Doi O, Kodama K, Yokouchi H, Tateishi R. (1992) An evaluation of the prognostic significance of alpha-1-antitrypsin expression in adenocarcinomas of the lung: an immunohistochemical analysis. *Br. J. Cancer*. 65:300–2.
31. Vercaigne-Marko D, Carrere J, Guy-Crotte O, Figarella C, Hayem A. (1989) Human cationic and anionic trypsins: differences of interaction with alpha 1-proteinase inhibitor. *Biol. Chem. Hoppe Seyler*. 370:1163–71.
32. Yousef GM, *et al*. (2003) The human kallikrein protein 5 (hK5) is enzymatically active, glycosylated and forms complexes with two protease inhibitors in ovarian cancer fluids. *Biochim. Biophys. Acta*. 1628:88–96.
33. Tseng IC, *et al*. (2008) Purification from human milk of matriptase complexes with secreted serpins: mechanism for inhibition of matriptase other than HAI-1. *Am. J. Physiol. Cell Physiol*. 295:C423–31.
34. Petrache I, *et al*. (2006) Alpha-1 antitrypsin inhibits caspase-3 activity, preventing lung endothelial cell apoptosis. *Am. J. Pathol*. 169:1155–66.
35. Lewis EC. (2012) Expanding the clinical indications for α(1)-antitrypsin therapy. *Mol. Med*. 18:957–70.
36. Louie SG, Sclar DA, Gill MA. (2005) Aralast: a new alpha1-protease inhibitor for treatment of alpha-antitrypsin deficiency. *Ann. Pharmacother*. 39:1861–9.
37. Yang S, *et al*. (2011) Pancreatic cancers require autophagy for tumor growth. *Genes. Dev*. 25:717–29.
38. Rubinsztein DC, Codogno P, Levine B. (2012) Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat. Rev. Drug. Discov*. 11:709–30.
39. Kagawa S, *et al*. (2001) Deficiency of caspase-3 in MCF7 cells blocks Bax-mediated nuclear fragmentation but not cell death. *Clin. Cancer Res*. 7:1474–80.
40. Huang H, *et al*. (2004) Alpha1-antitrypsin inhibits angiogenesis and tumor growth. *Int. J. Cancer*. 112:1042–8.