Biochimica et Biophysica Acta 1783 (2008) 2294–2300

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Role of the proteolytic hierarchy between cathepsin L, cathepsin D and caspase-3 in regulation of cellular susceptibility to apoptosis and autophagy

Xin Zheng [a], Fei Chu [a], Bernard L. Mirkin [a,b], Thangirala Sudha [c], Shaker A. Mousa [c], Abdelhadi Rebbaa [c,*]

[a] Children's Memorial Research Center, Children's Memorial Hospital, Department of Pediatrics, The Feinberg School of Medicine, Northwestern University, Chicago, IL, USA
[b] Department of Molecular Pharmacology and Biological Chemistry, The Feinberg School of Medicine, Northwestern University, Chicago, IL, USA
[c] The Pharmaceutical Research Institute at Albany, Albany College of Pharmacy, 1 Discovery Drive, Room 238, Rensselaer, NY 12144, USA

ARTICLE INFO

Article history:
Received 17 March 2008
Received in revised form 21 July 2008
Accepted 28 July 2008
Available online 13 August 2008

Keywords:
Cathepsin L
Cathepsin D
LC3
Apoptosis
Autophagy

ABSTRACT

The present investigation was undertaken to measure the relative abilities of pro-death *versus* pro-survival proteases in degrading each other and to determine how this might influence cellular susceptibility to death. For this, we first carried out *in vitro* experiments in which recombinant pro-death proteases (caspase-3 or cathepsin D) were incubated with the pro-survival protease (cathepsin L) in their respective optimal conditions and determined the effects of these reactions on enzyme integrity and activity. The results indicated that cathepsin L was able to degrade cathepsin D, which in turn cleaves caspase-3, however the later enzyme was unable to degrade any of the cathepsins. The consequences of this proteolytic sequence on cellular ability to undergo apoptosis or other types of cell death were studied in cells subjected to treatment with a specific inhibitor of cathepsin L or the corresponding siRNA. Both treatments resulted in suppression of cellular proliferation and the induction of a cell death with no detectable caspase-3 activation or DNA fragmentation, however, it was associated with increased accumulation of cathepsin D, cellular vaculolization, expression of the mannose-6-phosphate receptor, and the autophagy marker LC3-II, all of which are believed to be associated with autophagy. Genetic manipulations leading either to the gain or loss of cathepsin D expression implicated this enzyme as a key player in the switch from apoptosis to autophagy. Overall, these findings suggest that a hierarchy between pro-survival and pro-death proteases may have important consequences on cell fate.

1. Introduction

Protein degradation is as important for cellular viability as their synthesis and proteases have long been considered as guardians of cellular homeostasis. A number of key cellular functions are controlled by different proteases such as the execution of cell death mediated by caspases [1], degradation and elimination of abnormal proteins by cathepsins or proteasome [2], and cancer metastasis facilitated by metalloproteases and cathepsins [3]. Due to their protein nature, it is likely that enzymes from one group may exhibit cleavage sites for proteases from another group. Consequently, cellular functions that are directly controlled by the first group of proteases would be indirectly governed by the second one. It has been shown for instance that cathepsin D can cleave and activate the pro-apoptotic molecule Bid, which in turn causes cytochrome c release and the activation of caspase-3 [4]. Based on this, cathepsin D may be considered as a pro-apoptotic molecule although it has been recently shown that this enzyme may also signal for a non-apoptotic cell death [5]. In contrast to the pro-apoptotic proteases caspase-3 and cathepsin D, cathepsin L was reported to play a protective role against stress-mediated cell death [6]. An example for this was provided by previous work demonstrating that this enzyme attenuates cellular susceptibility to apoptosis by degrading cathepsin D [7]. The role of cathepsin L in cell survival was demonstrated in *C. elegans* in which transfection with siRNAs to this enzyme resulted in embryonic lethality [8]. Although the loss of cathepsin L expression was not lethal in mice, it was associated with severe abnormalities in the heart, brain, and skin [9–12], suggesting that this enzyme may be required for cell survival and normal organ development. In agreement with this concept, we have reported that cathepsin L protects cancer cells from the cytotoxic effect of chemotherapeutic agents and its inhibition reverses drug resistance in various cancer cell lines [13]. Findings from this study indicated that that cathepsin L protects cancer cells not only against drug induced apoptosis but also against irreversible growth arrest, termed senescence [13], suggesting that targeting this molecule may hold great promise for therapeutic interventions against cancer.

The possibility that pro-death and pro-survival proteases may coexist and interact within the same cellular compartment raises the possibility that they will regulate the function of each other. As a result, cellular physiological functions and particularly its susceptibility to death will be significantly altered. The present study was

* Corresponding author. Tel.: +1 518 694 7560; fax: +1 518 694 7567.
E-mail address: rebbaaa@acp.edu (A. Rebbaa).

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doi:10.1016/j.bbamcr.2008.07.027

carried out to test this hypothesis and define the type of cellular response that may result from disruption of protease hierarchy.

2. Material and methods

Human neuroblastoma SKN-SH, osteosarcoma SaOS2 and prostate cancer LNCap cells were purchased from ATCC (Rockville MA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from BioWhittaker (Walkersville, MD). The following drugs and reagents were obtained from the companies cited: recombinant cathepsin L, cathepsin D and caspase 3 from R&D Systems (Minneapolis, MN), cathepsin L inhibitor Napsul-Ile-Trp-CHO (iCL) from Biomol (Playmouth Meeting, PA); the autophagosome inhibitor 3-MA and doxorubicin (Sigma, St. Louis, MO); Antibody to cathepsin L (Novus Biologicals, Littleton, CO); cathepsin D (Abcam, Cambridge, MA); active caspase-3 (Cell signaling Technology, Beverly, MA), p21/WAF1, LC3-II (Santa Cruz Biotechnologies, Santa Cruz, CA) and MPR (Abcam, Cambridge, MA); reagents for siRNA transfection from (Gene Therapy Systems, San Diego, CA), antibody to beta-Actin from Sigma (St. Louis, MO); secondary antibodies conjugated to horseradish peroxidase from BioRad (Hercules, CA); Enhanced chemiluminescence reagents (ECL) from Amersham (Arlington Heights, IL); immobilon-P transfer membrane for western blots from Millipore (Bedford, MA).

2.1. Cleavage reactions

Cleavage of recombinant cathepsin D and caspase-3 by purified cathepsin L was performed by incubating various amounts of the latter enzyme (1 to 5 ng) with 100 ng of the substrates (cathepsin D and caspase-3) in reaction buffer (100 mM sodium acetate pH 5, 1 mM EDTA, and 4 mM dithiothreitol). Similar procedure was undertaken to study the cleavage of cathepsin L and caspase-3 by cathepsin D in 100 mM sodium acetate, pH 3. The cleavage of both cathepsins by caspase-3 was carried out in a reaction buffer (100 mM HEPES, 20% (v/v) glycerol, 0.5 mM EDTA, 5 mM DTT). After incubation for 20 min at room temperature, Proteins were denatured by boiling, separated by SDS-PAGE and transferred to Immobilon P membrane. Specific antibodies were used to detect each one of these enzymes.

2.2. Protease activity assays

Cathepsin L activity was measured as described previously [13]. Briefly, the purified enzyme (200 ng) was incubated in a 96-well plate for 15 min at room temperature in 100 μl of reaction buffer [100 mM sodium acetate (pH 5), 1 mM EDTA, and 4 mM DTT]. One hundred μl of substrate were added and incubated for an additional 30 min at room temperature. Fluorescence was measured in a plate reader (Victor Multilabel Counter; Perkin-Elmer) at 380-nm excitation and 450-nm emission wavelengths. Cathepsin D activity was measured using the assay kit #143-100 (Biovision, Mountain View, CA) as described by the manufacturer. The activity of caspase-3 was assayed using the caspase-specific fluorescence peptide substrates Ac-DEVD-AFC (Biomol Playmouth Meeting, PA) according to the manufacturer's instructions. Relative enzymatic activities were obtained by comparing those of enzymes incubated in reaction buffer alone to those in the presence of other proteases.

2.3. Western blot

Cells were seeded in DMEM containing 10% FBS, and after 24 h, increasing amounts of iCL were added to the culture medium and incubated for an additional 24 h. Western blots were carried out as described earlier [22]. Proteins of interest were identified by reaction with specific primary and secondary antibodies linked to horseradish peroxidase and detected by chemiluminescence.

2.4. Electron and fluorescence microscopy

Cells were seeded in 75 cm^2 flaks and incubated for 24 h at 37 °C then subjected to treatment with iCL for an additional 24 h. For electron microscopy, the cells were were trypsinized and washed three times with cold PBS, fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, and dehydrated and embedded in epon. Thin sections were cut and following lead to citrate and uranyl-acetate contrasting, the cells were observed under electron microscope and photographs taken. For fluorescent microscopy, the cells were fixed with paraformaldehyde 4% for 5 min and after washing, they were incubated with antibodies against LC3-II at 4 °C for 15 h. After wash with PBS, the cells were then incubated for 1 h at 4 °C with fluorescence labeled secondary antibodies and pictured under fluorescence microscopy.

2.5. DNA fragmentation

Cells were incubated with iCL at various concentrations for 24 h at 37 °C and DNA prepared from Triton X-100 lysates for analysis of fragmentation. Briefly, cells were lysed in a hypotonic solution containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 0.2% Triton X-100, and centrifuged at 11000 ×*g* for 5 min. Supernatants were electrophoresed on a 1% agarose gel and DNA fragments visualized under UV light after staining with ethidium bromide.

2.6. APO-BRDU assay for apoptosis

SaOS2 cells (1×10^6) were plated in 100 mm dishes and allowed to adhere for 24 h. Cells were treated with the appropriate concentrations of cathepsin L inhibitor. After 24 h, the cells were harvested in PBS and fixed in 1% paraformaldehyde. After two washes in PBS, cells were re-suspended in ethanol and stored at −20 °C until further analysis. Percentage apoptosis was determined using the APO-BRDU assay kit from BD Biosciences (CA, USA). In this assay, the cell pellet was incubated with brominated deoxyuridine triphosphate (BRDU) and TdT (terminal deoxynucleotidyl transferase) enzyme for 24 h at 28 °C. BRDU incorporated into the 3′-hydroxyl termini of double- and single-stranded DNA was identified by staining the cells with a fluorescent labeled anti-BRDU monoclonal antibody, using flow cytometry.

2.7. siRNA design and transfections

The Human cathepsin D and cathepsin L siRNAs [14] were synthesized by Dharmacon (Lafayette, CO) and transfected according to the manufacturer's protocol. Cathepsin D gene construct (Origen, Rockville, MD) was transfected using lipofectamin as described by the manufacturer. Twenty four hours after transfection, the cells were treated with iCL and maintained in culture for an additional 48 h before measure of cell viability and expression of the silenced molecules by Western blot.

3. Results

3.1. Analysis of proteolytic reactions between cathepsin L, cathepsin D and caspase-3 in vitro

Active forms of the enzymes were incubated in their corresponding reaction buffers with the remaining enzymes used as potential substrates and at the end of the proteolysis reaction, Western blots using specific antibodies were carried out to assess the extent of cleavage. As shown in Fig. 1 (panel A), incubation of cathepsin L with either cathepsin D or caspase-3 resulted in a sharp decrease in the amount of the latter two enzymes while the level of cathepsin L itself was not affected. This data suggests that cathepsin L was able to

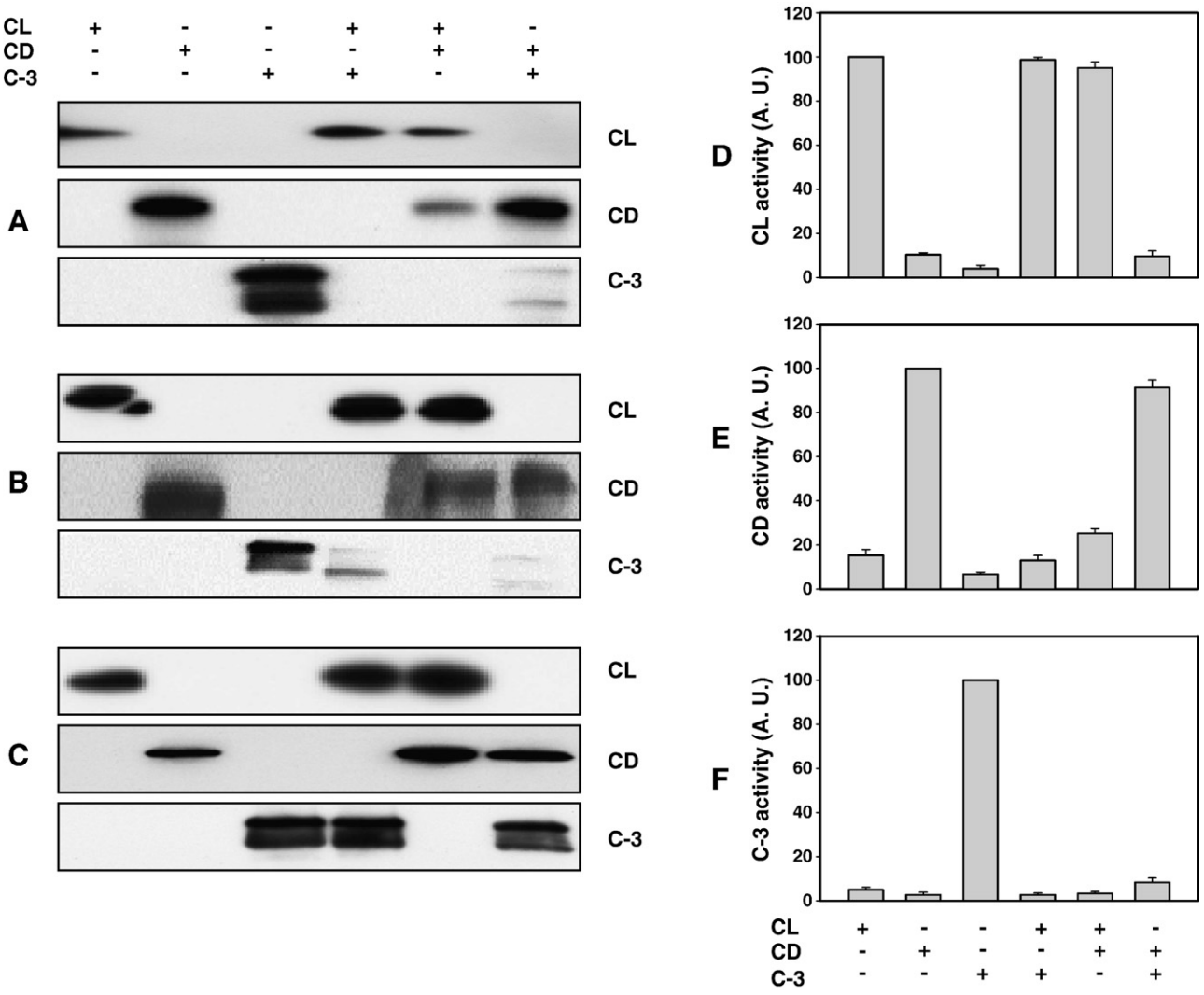

Fig. 1. Proteolytic interactions between cathepsin L (CL), cathepsin D (CD) and caspase-3 (C-3). Recombinant cathepsin L (panel A), cathepsin D (panel B) and caspase-3 (panel C) were respectively incubated in the corresponding buffers under optimal conditions with each one of the remaining enzymes used as substrates. At the end of reaction, protein mixtures were separated by electrophoresis and individual enzymes detected with specific antibodies. The activity of individual enzymes, cathepsin L (CL, panel D), cathepsin D (CD, panel E) and caspase-3 (C-3, panel F), were measured *in vitro* at the end of reactions described in panel A. Protease activities are represented in arbitrary units (AU). The data represent means of three determinations ± SE.

cleave both cathepsin D and caspase-3 but was not cleaved by them. Of note, cathepsin D was able to degrade caspase 3 in cathepsin L reaction buffer however caspase-3 was unable to cleave any of the cathepsins under these conditions. In agreement with these proteolytic reactions, respective enzyme activities (Fig. 1, panels D–F) correlated with the protein levels detected in panel A, indicating that proteolysis of any of these enzymes is likely to translate into loss of activity.

When experiments similar to those described in Fig. 1 (panel A) were carried out under optimal conditions for cathepsin D (Fig. 1, panel B), caspase-3 was degraded by both cathepsins, however, none of these enzymes was hydrolyzed suggesting that they may have superior proteolytic capability than caspase-3. This is confirmed by the finding that caspase-3, even under conditions optimum for its activation, was unable to degrade cathepsin L or D as shown in Fig. 1 (panel C). Similar experiments to those described in panel D, E and F regarding the correlation between protein amounts, detected by Western blot in panel B and panel C, and the corresponding enzymatic activities were carried out and the results confirmed this correlation (data not shown). Together, the data presented in Fig. 1 suggest that cathepsin L is the strongest among the three proteases tested, therefore, its targeting is likely to influence intracellular levels of caspase-3 and susceptibility apoptosis.

3.2. Characterization of cellular response associated with cathepsin L inhibition

Taking into account the demonstrated role of cathepsin L in cell survival [13], the data described above (Fig. 1) suggests that specific inhibition of this enzyme may result a cell death characterized by increased accumulation of cathepsin D and decreased caspase-3. To verify this hypothesis, we analyzed the cellular toxic response caused by incubation with increasing amounts of cathepsin L inhibitor (iCL). As shown in Fig. 2A, this compound induced a dose dependent suppression of SaOS2 cell proliferation with a maximum effect at 40 μM. Morphologically, only small changes were noticed at low and intermediate drug concentrations (10 and 20 μM). However when cells were treated with 40 μM, cytoplasmic vacuoles were formed and the cells become round before undergoing death (Fig. 2B). Electron microscopy revealed noticeable changes particularly in the cytoplasm of cells treated with iCL at 40 μM. The most obvious changes were extensive vacuolization and the presence of double membrane

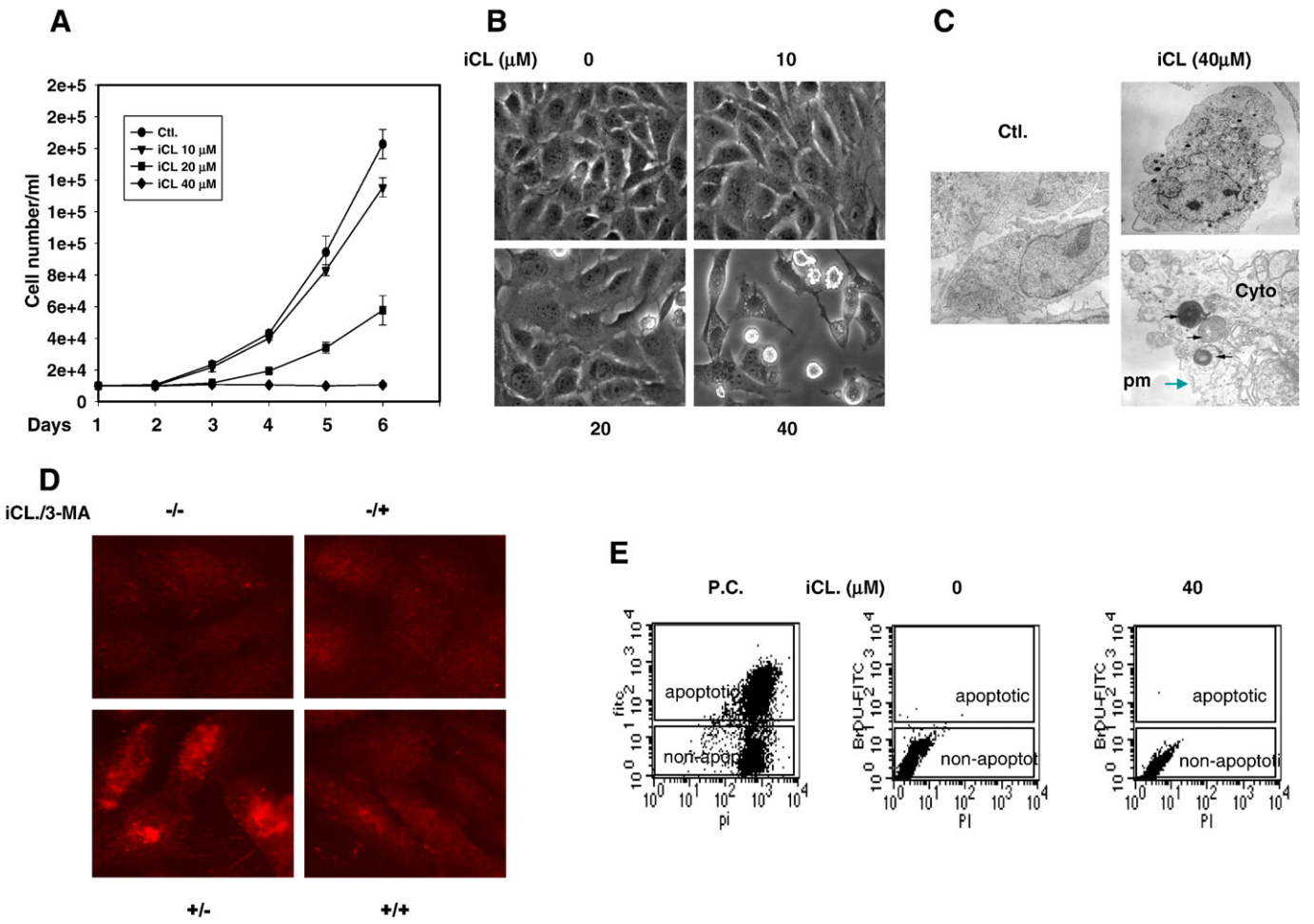

Fig. 2. Cellular response to cathepsin L inhibition. (Panel A) SaOS2 cells were incubated in 24 well plates with increasing concentrations of cathepsin L inhibitor (μM) and cell number counted for up to 6 days. (Panel B) Morphological changes associated with iCL treatment. (Panel C) Ultra-structural modifications induced by iCL at the cellular level. Note that at 40 μM, iCL induced cytoplasmic vacuolization and the appearance of double membrane vesicles containing undigested material indicative of autophagic death (ctl: control., Cyto: cytoplasm., pm: plasma membrane). (Panel D) SaOS-2 cells were treated with iCL (40 μM) in the absence or the presence of 3-MA for 24 h. LC3-II staining was detected by fluorescence microscopy. A representative staining from three independent experiments is shown. (Panel E) SaOS2 cells were treated as in panel D with iCL for 24 h, then fixed and analysed by flow cytometry to measure apoptosis. (P.C: positive control where the cells were exposed to doxorubicin 10^{-6} M for 24 h).

vesicles, some of which may contain indigested material (panel C). In additionto this, fluorescence microscopy (Fig. 2D) demonstrated increased amounts of the autophagic marker LC3-II in cells treated by 40 μM of iCL, and that expression of this marker was suppressed by the autophagosome inhibitor 3-MA (Fig. 2D). Flow cytometry analysis indicated that cathepsin L inhibitor at 40 μM did not induce apoptotic death (Fig. 2E). Together, these features are reminiscent of autophagic cell death [15–17].

3.3. Molecular determinants associated with cellular response to cathepsin L inhibition

We have analyzed the expression of molecular markers known to be associated with autophagy such as cathepsin D, the mannose 6-phosphate receptor (MPR) and LC3-II [15,18] and compared their expression to that of the cell cycle inhibitor p21/WAF1, a marker of proliferation arrest [19], and also to that of caspase-3 activation. As shown in Fig. 3A, exposure to low concentrations of iCL was associated with an increased expression of p21/WAF1 in agreement with our previous findings that inhibition of cathepsin L [13] forces cancer cells into senescence. Cathepsin D expression increased in a dose dependent manner and particularly in response to high concentrations of iCL, confirming the observation made earlier that this enzyme could be a substrate for cathepsin L *in vivo*. Expression of MPR also increased in a manner that correlates cathepsin D, reaching a maximum at 40 μM of iCL, and thus seems to correlate with the onset of autophagy observed in Fig. 2B–D. Moreover, the light chain of LC3 (LC3-II) accumulated at this iCL concentration (Fig. 3A), further confirming the autophagic nature of this cellular response.

Analysis of caspase-3 cleavage in cells treated with iCL (Fig. 3B) led to the unexpected finding that at (40 μM), this compound caused a unique proteolytic profile of this enzyme with the appearance of a band at about 25 KD termed CLIF (for Cathepsin L inhibition-Induced Fragment). As shown in this figure, the p19 and p17 fragments that are usually generated in response to apoptosis-inducing stressors such as doxorubicin, represent minor fractions of the total cleavage products in response to cathepsin L inhibition. This data suggests that apoptosis may represent only a minor component of cell death induced by cathepsin L inhibitor and that autophagy may indeed be the dominant response to this treatment.

The role of CLIF in mediating autophagy in this system is not yet clear, however, we found that in cells treated with doxorubicin to activate caspase-3, cathepsin L inhibition reduced the level of the active enzyme and enhanced the levels of CLIF in a dose dependent manner (Fig. 3C). Since this appeared to correlate with expression

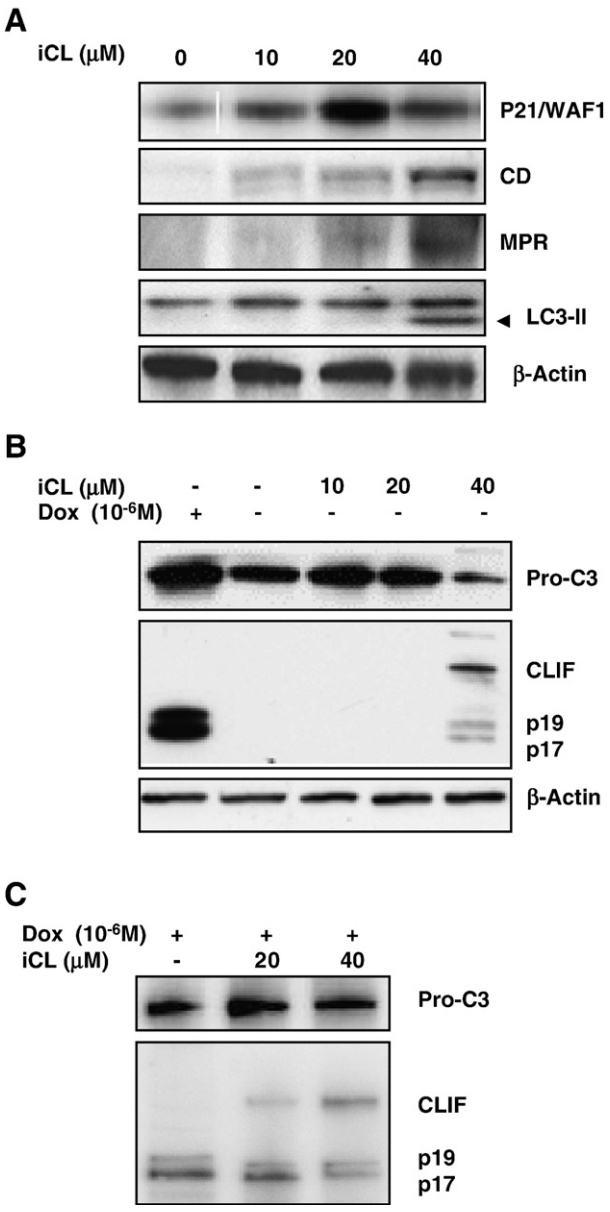

Fig. 3. Molecular determinants associated with cathepsin L inhibition. (Panel A) Cells were treated with increasing iCL concentrations for 24 h and expression of p21/WAF1, cathepsin D (CD), Manose-6-phosphate receptor (MPR) and LC3-II (arrow) was detected by Western blot using specific antibodies. Antibody to β-actin was used as a loading control. (Panel B) SaOS2 cells were treated with doxorubicin (10^{-6} M) or with iCL at the indicated concentrations for 24 h and the proteolytic cleavage of pro-caspase-3 (Pro-C3) into the cells was analyzed by Western blot. Antibody to beta actin (β-actin) was used as a loading control. CLIF (Cathepsin L Inhibition-induced Fragment). (Panel C) Analysis of iCL induction of CLIF: SaOS2 cells were treated with doxorubicin 10^{-6}M in the absence or the presence of iCL at 20 or 40 μM. After incubation for 24 h, Western blot was carried out to analyze expression of active caspase-3 (fragments p17 and p19) and CLIF.

cathepsin D (Fig. 3B) which is known to cleave caspase-3, CLIF may represent a product of degradation of caspase-3 by cathepsin D.

3.4. Respective roles of cathepsin L and D in mediating cellular response to iCL

To determine whether cellular response to iCL was indeed caused by specific inhibition of cathepsin L, we transfected cells with siRNA oligonucleotides to knockdown expression of this enzyme, and analyzed expression of the molecular markers associated with proliferation arrest, autophagy and apoptosis. We have found that increased amounts of cathepsin L siRNA inhibited cellular proliferation in a dose dependent manner (Supplemental data S1), highlighting the essential role of this enzyme in cellular protection. Also, in a manner that was inversely proportional to decreased cathepsin L expression (Fig. 4A), those of p21/WAF1 and cathepsin D increased. Interestingly, no caspase-3 activation or DNA fragmentation were detected under these conditions (Fig. 4A and Supplemental data S1), confirming the findings described above that a loss of cathepsin L function may cause a non-apoptotic cell death. Morphological and immunostaining analyses further confirmed that siRNA to cathepsin L induces autophagy as the transfected cells displayed increased vacuolization and LC3-II staining (Fig. 4B).

The role of cathepsin D in mediating iCL-induced autophagy and its association with CLIF were investigated by inducing gain and loss of expression of this enzyme. As shown in (Fig. 4C), cathepsin D gene-tranfected cells had elevated amounts of the enzyme as compared to non-transfected cells or those transfected with the empty plasmid. As it would be expected, iCL induced the expression of cathepsin D in the non-transfected cells, and to a much stronger extent in the transfected ones (Fig. 4C). Cellular vacuolization was also enhanced in cathepsin D-transfected cells in the presence and in the absence of iCL (Fig. 4D). We have noted that when cells were treated with 40 μM iCL, they become round and detach, rendering pictures difficult to take (data not shown). Inversely, iCL-induced cathepsin D expression was completely suppressed in cells transfected with the corresponding siRNA (Fig. 4E). This was associated with reduced vacuolization induced by iCL (Fig. 4F). Interestingly, CLIF was also associated with the induction of cathepsin D and the onset of autophagy (Fig. 4C–F) suggesting that expression of this caspase-3 isoform is directly related to cathepsin D and that the two molecules may play key roles in mediating the autophagic effect of iCL.

In summary, our data suggests that cathepsin L may exert its pro-survival function by degrading the pro-death proteases cathepsin D and caspase 3. We also show that inhibition of cathepsin L does not necessarily enhance cellular susceptible to apoptosis, particularly in cells that express cathepsin D. While moderate inhibition of cathepsin L enhanced expression of the cell cycle inhibitor p21/WAF1 resulting in proliferation arrest, severe inhibition of this enzyme resulted in enhanced accumulation of cathepsin D and depletion of cellular pools of caspase 3, thereby causing a switch from apoptosis to autophagy. Based on this, it is suggested that depending on cathepsin L inhibition level, cancer cell may undergo either proliferation arrest or autophagic cell death. This later demise appears, at least in our cellular models, to be associated with the accumulation of cathepsin D and CLIF.

4. Discussion

For more than a decade, apoptosis has taken the central stage as the most relevant biological response of cancer cells to a variety of treatments, consequently, elements of the associated pathways were often utilized for a rational design of new anti-cancer therapeutics. However not all cancer cells are susceptible to apoptosis and evidence is now accumulating that in response to chemotherapeutic agents, they may activate alternative pathways leading either to irreversible growth arrest (senescence) or non-apoptotic types of cell death such as necrosis, mitotic catastrophe, and autophagy [20,21]. Moreover, it has been demonstrated that inhibition of apoptosis may result in a switch to senescence, necrosis or other undefined cell deaths [22–24], highlighting the need for identification of molecular mechanisms that regulate these switches. The present study investigated the existence of a proteolytic hierarchy between pro-survival and pro-death proteases, and its role in regulating cellular decisions between survival, apoptosis and autophagy. This was addressed by measuring the ability of caspase-3 (implicated in apoptosis), cathepsin D (implicated in both apoptosis and autophagy), or cathepsin L (implicated in cell survival) to neutralize the other two proteases,

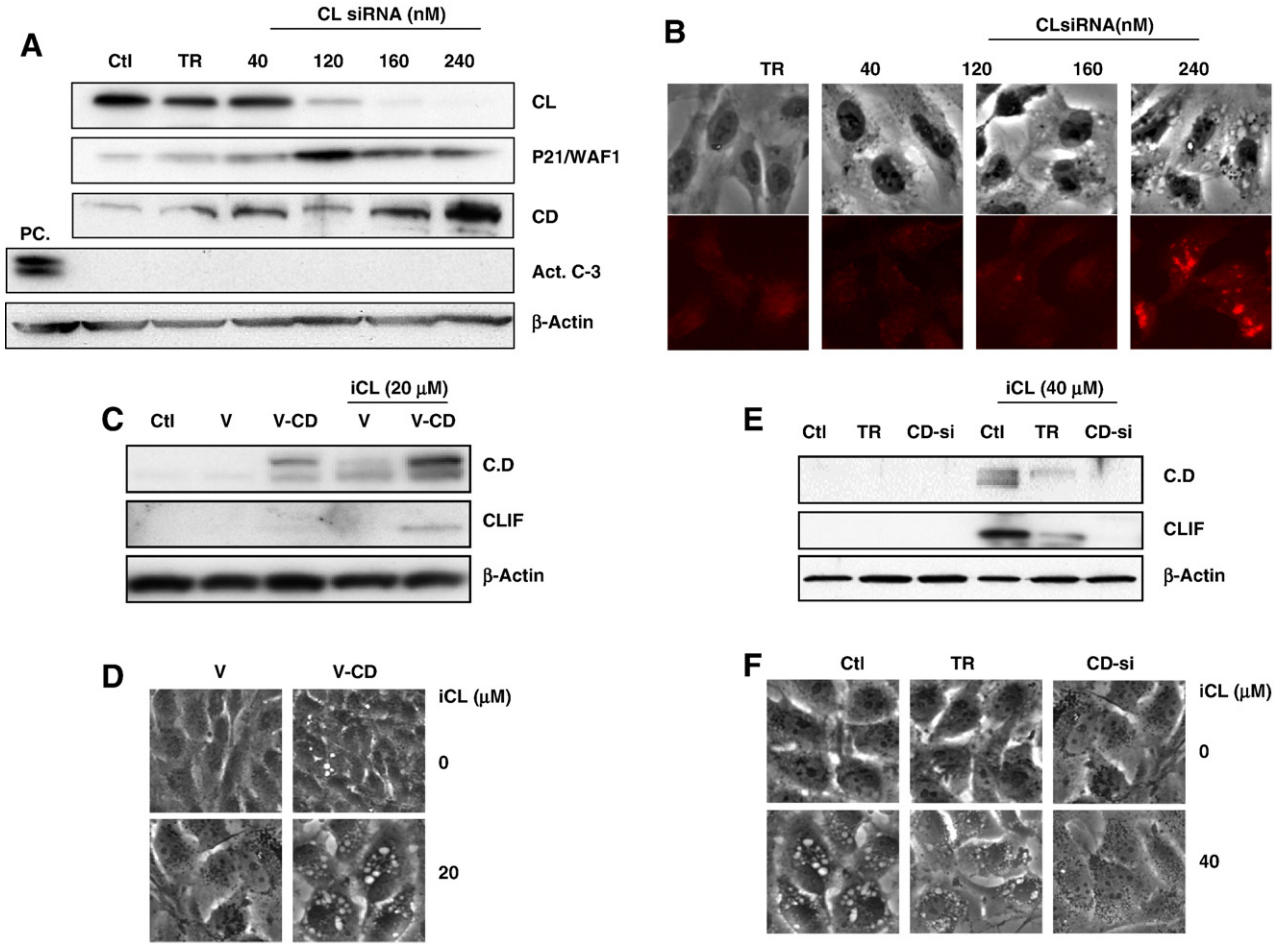

Fig. 4. Respective roles of cathepsin L and cathepsin D in mediating cellular response to iCL. (Panel A) SaOS2 cells were non-transfected (Ctl), treated with the transfection reagent alone (TR), or transfected with increasing amounts of siRNA to cathepsin L (CLsiRNA). Expression of cathepsin L and other molecular markers of proliferation arrest (p21/WAF1), apoptosis (active caspase-3, Act. C-3), and autophagy (cathepsin D, CD) were analyzed in siRNA-transfected and non-transfected cells by Western blot using specific antibodies. Positive control (PC) represents SKN-SH cells treated with doxorubicin 10^{-6} M for 24 h. (Panel B) The cells were treated as above (panel B) and cellular morphology was analyzed underlight microscopy to detect vacuols (upper panel). Staining for LC3-II was visualized by confocal microscopy. (Panel C) SaOS2 cells non-transfected (Ctl) transfected with the empty vector alone (V) or with the vector containing cathepsin D construct (V-CD) were analyzed by Western blot for expression of this enzyme in the presence or the absence of iCL (20 μM) as described above. Expression of CLIF was also analyzed under these conditions. Antibody to β-actin was used as a loading control. (Panel D) Light microscopy photographs showing cellular vacuolization in cells transfected with the vector alone (V) or with the vector harboring cathepsin D construct (V-CD) in the absence or in the presence of iCL (20 μM). (Panel E) effect of siRNA to cathepsin D on expression of the enzyme and that of CLIF. SaOS2 cells were transfected wither with a vehicle transfection reagent alone (TR) or siRNA to cathepsin D (CD-si). Expression of both cathepsin D and CLIF were analysed by Western blot. Antibody to beta actin (β-Actin) was used as a loading control. (Panel F) Light microscopy photographs showing cellular vacuolization in cells non-transfected (Ctl), transfected with the reagent alone (TR) or with the reagent containing cathepsin D siRNA (CD-si) and this in the absence or in the presence of iCL (40 μM).

and determination of how this might effect cellular susceptibility to death. The data shown in Fig. 1 provided evidence in support of this concept and suggested that the pro-survival, cathepsin L, located at the top of this proteolytic cascade, is likely to play a key role in regulating the levels of cathepsin D and caspase-3 as well as the cellular processes associated with them.

The protease cascade described here also provided further support for the pro-survival function of cathepsin L and suggest that this may be due, at least in part, to its ability to deplete the cells from the pro-apoptotic molecule caspase-3. However, since cathepsin L inhibition causes the accumulation of cathepsin D (a caspase-3 hydrolase), the ensuing death would be associated with cathepsin D and not with caspase 3. The data shown in Fig. 2 are in support of this hypothesis as cellular treatment with increasing amounts of iCL culminated in induction of autophay. Expression of several morphological and molecular markers indeed confirmed this observation. These include increased cellular vacuolization (Fig. 2B), the accumulation of autophagosomes (Fig. 2C), LC3-II staining and its inhibition by 3-MA (Fig. 2D), and the absence of apoptotic death (Fig. 2E). Also, Western blot analysis of cells subjected to treatment with increased concentration of cathepsin L inhibitor induced a number of molecular autophagic markers (Fig. 3A), providing further evidence that autophagy is the primary type of death induced by this approach. Curiously, the lack of caspase-3 activation is compensated for by the appearance of high molecular weight isoform of this enzyme (CLIF), that correlated with the level of cathepsin D expression in iCL treated cells (Figs. 3C, 4C and E). The actual role of CLIF as a mediator of autophagic death or as a cellular marker of this cellular demise remains to be elucidated.

The specificity of cathepsin L inhibition in triggering autophagy was verified by inhibiting its expression with siRNAs (Fig. 4), which resulted in enhanced accumulation of molecular determinants associated with this cell death (i.e. cathepsin D and LC3-II) in the transfected cells. These findings (Fig. 4A) further confirmed the observation made earlier that caspase-3 was not activated by such treatment and that the cells may have died primarily from autophagy. We speculate that the lack of caspase-3 activation in response to cathepsin L inhibition may be caused by increased degradation by cathepsin D, suggesting that the latter enzyme could be responsible for switching cell death from apoptosis to autophagy. The results presented in Fig. 4C–F are in favor

of this possibility since genetic manipulations leading to either loss or gain of cathepsin D function affected cellular ability to undergo autophagy. Based on this, cathepsin D may represent a conceivable target to manipulate cellular decision to undergo autophagy.

In conclusion, the present study provided evidence for the existence of a proteolytic hierarchy between pro-survival and pro-death proteases and suggested that changes in cellular proteolytic profiles are likely to have significant consequences not only on its survival but also on the type of death it may undergo in response to a specific stress. Comprehensive investigation of cellular protease signature and proteolytic hierarchy is warranted as this may open new avenues for the design of approaches to better control cellular fate in normal and diseased tissues.

Acknowledgements

This study is supported in part by the National Cancer Institute (1R01 CA096616-01A1 to A.R.), the Illinois Department of Public Aid (A.R.), and NCI 1R41 CA128152-01 to (S.A.M). The authors wish to thank Patricia Phillips for assistance in the preparation of this manuscript. This work is dedicated to the memory of Dr. Bernard L. Mirkin.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.07.027.

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