Proteasome inhibition-induces endoplasmic reticulum dysfunction and cell death of human cholangiocarcinoma cells

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Abstract

AIM: To determine if proteasome inhibition induces apoptosis in human cholangiocarcinoma cells, and if so, to elucidate the cellular mechanisms.

METHODS: Studies were performed in the human KMCH, KMBC, and Mz-ChA-1 cholangiocarcinoma, and normal rat cell lines. MG132, a peptide aldehyde, which inhibits the chymotrypsin-like activity of the proteasome was employed for this study. Apoptosis was assessed morphologically by 4'-6-Diamidino-2-phenylindole (DAPI) nuclear staining and fluorescence microscopy. Mitochondrial membrane potential was examined using a fluorescent unquenching assay. Ultrastructural changes during cell death were examined using transmission electron microscopy (TEM). Caspase 3/7 activity was assessed using an enzymatic-based fluorescent assay. Cytosolic-free calcium concentrations were measured using Fura-2 and digitized fluorescent microscopy.

RESULTS: MG132, a proteasome inhibitor, induced apoptosis in all the cholangiocarcinoma cell lines examined. In contrast, minimal cytotoxicity was observed in normal rat cholangiocytes. Apoptosis was time- and -concentration-dependent. There was no change in the mitochondrial membrane potential between treated and untreated cells. Ultrastructural examination by transmission electron microscopy displayed the classic features of apoptosis, but in addition, there was also dramatic vacuolization of the endoplasmic reticulum (ER). Unexpectedly, no increase in caspase 3/7 activity was observed in MG132 treated cells, nor did the pancaspase inhibitor, Q-VD-OPh prevent cell death. The protein synthesis inhibitor, cycloheximide, blocked apoptosis induced by proteasome inhibitor indicating that ER dysfunction was dependent upon the formation of new proteins.

CONCLUSION: Proteasome inhibition induces ER

dysfunction and caspase-independent cell death selectively in human cholangiocarcinoma cells. Proteasome inhibitors warrant evaluation as anticancer agents for the treatment of human cholangiocarcinoma.

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Key words: MG132; Cholangiocarcinoma; Proteasome; Apoptosis; Calcium

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INTRODUCTION

Cholangiocarcinoma is a malignant disease arising from the epithelial cells, termed cholangiocytes, lining the intra and extrahepatic bile duct. Unfortunately, the incidence of disease is increasing in many Western countries^[1,2]. Surgery and liver transplantation are the only curative treatment options; however, disease recurrence is common even after ostensibly curative surgical procedures^[3,4]. Non-surgical therapy is palliative and there is no proven medical therapy for this neoplasm. Thus, cholangiocarcinoma is a devastating disease which frequently causes death. Additional therapies are needed for the treatment of this cancer. Cholangiocarcinoma has many phenotypic similarities with the hematologic malignancy multiple myeloma. For example, both neoplasms are IL-6 dependent, evade apoptosis by overexpression of the Bcl-2 family protein Mcl-1, and are associated with genetic silencing of the tumor suppressor gene *p16*^[5,6]. Recently, targeted inhibition of the proteasome has been shown to be effective anticancer therapy for multiple myeloma^[7]. In contrast, the effect of proteasome inhibition as therapy for cholangiocarcinoma is unknown.

Proteasomes are complex macromolecular structures which enzymatically degrade ubiquitinated proteins^[8]. The barrel shaped 20S proteasome is a very large ATP dependent proteolytic complex which has 4 rings that enclose a central hollow where proteolysis takes place^[9]. Its 2 central β -rings contain multiple chymotrypsin-like, two trypsin-like and two caspase-like activities. The outer 2 alpha rings encircle a small opening through

which polypeptide substrates enter. These alpha rings are essential for assembly of the whole particle. The ubiquitin-proteasome pathway plays an important role in signal transduction, transcriptional regulation and response to stress^[10,11]. Inhibition of this pathway can trigger cell cycle arrest at G1-S and G2-M phases of the cell cycle and apoptotic pathways^[12]. Although proteasome inhibition may be predicted to have nonspecific cytotoxicity, malignant cells are much more sensitive to the proapoptotic effects of proteasome inhibition than normal cells^[13]. The potential explanation for this selectivity appears to be due to the fact that cancer cells have more defective proteins accumulating at much higher rates than normal cells. This increases their dependency on optimal proteasome function to dispose of these proteins. Proteasome failure leads to the accumulation of these proteins which are toxic to the cell.

In cancer cells, the accumulation of proteins, due to proteasome inhibition induces cell death by multiple mechanisms. Loss of proteasome function can lead to accumulation of pro-apoptotic mediators which trigger the cellular apoptotic machinery^[14,15]. For example proteasome inhibition leads to cellular accumulation of the proapoptotic BH3 domain only protein of the Bcl-2 family, Bim^[15]. Bim dependent cell death is associated with mitochondrial dysfunction and activation of intracellular caspases, cysteine proteases which trigger canonical apoptotic programs^[15]. By preventing activation of the pro-survival transcription factor, NF- κ B, proteasome inhibition can also trigger apoptosis^[16]. More recently, proteasome inhibitors have also been associated with cell death by inducing endoplasmic reticulum (ER) dysfunction^[17]. Thus, the mechanisms of cell death by proteasome inhibition are complex and likely cell type specific.

The overall objective of this study was to determine if proteasome inhibition induces apoptosis in cholangiocarcinoma cell lines. The results demonstrate that proteasome inhibition induces caspase-independent cell death of human cholangiocarcinoma cell lines. This cell death pathway is associated with structural abnormalities of the ER. Proteasome inhibition appears to induce ER dysfunction and cell death in human cholangiocarcinoma cells.

MATERIALS AND METHODS

Reagents

MG132 was from Calbiochem (San Diego CA). Bortezomib was obtained from the Mayo Clinic Formulary (Rochester, MN). DMEM (Life Technologies, Gaithersburg, MD), fetal bovine serum (Summit Biotechnology, Fort Collins, CO), Penicillin-streptomycin (Bio-Whittaker, Walkersville, MD), gentamycin (GIBCO BRL, Life Technologies, Grand Island, NY), and O-VDOPh (Enzyme Systems, Livermore, CA), ECL (Amersham, Arlington Heights, IL), BAPTA, AM (Invitrogen, Eugene, OR) staurosporine (Sigma Chemicals Co, St Louis, MO) and cycloheximide (Sigma Chemicals Co, St Louis, MO) were purchased as indicated.

Cell lines

The human cholangiocarcinoma cell lines KMCH-1,

KMBC, Mz-ChA-1, and normal rat cholangiocyte cell line were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin G (100 000 U/L), streptomycin (100 mg/L) and gentamycin (100 mg/dL) as described previously^[18-20]. The normal rat cholangiocyte cell line was a generous gift from Nicholas F. LaRusso, Mayo Clinic, Rochester, MN^[21]. Cells were maintained in a humidified incubator under an atmosphere containing 10% CO₂ at 37°C.

Quantitation of apoptosis

Morphologic features of apoptosis were quantified by assessing the characteristic nuclear changes of apoptosis (i.e., chromatin condensation and nuclear fragmentation) using the nuclear binding dye 4',6-diamino-2-phenylindole dihydrochloride (Sigma chemicals, St. Louis, MO) and fluorescent microscopy^[22].

Caspase 3/7 assay

Cells were cultured in 96 well plates (Corning, NY, NY). Cellular caspase activation was assessed using the Apo-One™ homogeneous caspase-3/7 Assay according to the supplier's instructions (the Apo-ONE™ Homogenous Caspase 3/7 activity kit-Promega Corporation, Madison WI, USA). The Apo-One™ Reagent was added directly to the cells in a 1:1 (Apo-One™ Reagent: cell culture medium) ratio of reagent to sample. After gently mixing by shaking at 300-500 r/min on a plate shaker for 30 s, the fluorescence of each well was measured at an excitation wave length of 485 and an emission wavelength of 530 nm using a fluorescent plate reader^[23].

Transmission electron microscopy

Cultured KMCH cells were fixed using Trump's fixative as previously described^[24]. Briefly, the media was aspirated and replaced with equal volume of Trump's fixative (1% glutaraldehyde and 4% formaldehyde in 0.1 mol/L phosphate buffer, pH 7.2). Specimens were post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer and embedded in Spurr's resin. Thin sections (70 nm) were cut and placed on copper grids and double stained with uranyl acetate and lead citrate. The tissue was examined and photographed with a transmission electron microscope (1200 EXII; JEOL, Peabody, MA) operating at 60 kV. Electron micrographs of cholangiocarcinoma cells each were examined by two masked observers.

Measurement of cytosolic free calcium (Ca²⁺)

Ca²⁺ was measured in cultured KMCH cells loaded with fura-2 using multiparameter digitized videomicroscopy as previously described by us^[25]. The microscope was a Zeiss IM-35 inverted fluorescence microscope (Thornwood, NY) equipped with phase contrast optics. A low light intensified video camera (Model 66, MTT-Dage, Michigan City, IN) collected fluorescent images which were digitized with a QVG/AFA-123 video acquisition and display board set (Imaging technologies, Woburn, MA) operating in a MicroPDP 11/23 computer (Digital Equipment Corporation, Maynard, MA). Ca²⁺ was quantified by ratio imaging of fura-2

fluorescence excited at 340 and 380 nm. Fluorescence was taken through a 395 nm dichroic reflector and a 470-550 emission filter. By using a K_d of 224 nmol/L for the fura-2- Ca^{2+} complex, the mean values of pixel ratios for each cell were converted to Ca^{2+} as described previously by Gyrinkiewicz *et al.*^[26]. $R_{\min}$, $R_{\max}$ and sfb2/sb2 values were calculated from measurements with fura-2 free acid solutions in capillary tubes (Vitro Dynamics, Inc., Rockaway, NJ) set on the microscope stage^[27]. To test whether the involvement of Ca^{2+} homeostasis could be the key to the specific vulnerability of cholangiocarcinoma cells to proteasome inhibition, we co-treated KMCH cell cultures with an intracellular Ca^{2+} chelator, BAPTA-AM (5 $\mu\text{mol/L}$) with MG132 for 24 h.

Quantification of mitochondrial membrane potential

The mitochondrial membrane potential in KMCH cells was measured by using a fluorescence unquenching assay as previously described in detail by us^[28]. This assay is based on the concept of resonance energy transfer between the mitochondrial membrane potential-sensitive dyes tetramethylrhodamine ethyl ester and mitotracker green. Cellular fluorescence was depicted using the multiparameter digitized fluorescence microscopy system described above and quantified by a software program (Universal Imaging Co) as previously described elsewhere in detail^[29].

Immunocytochemistry for cellular NF- κ B localization

Cells, cultured on collagen-coated glass cover slips, were fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.5% Triton-X in PBS and blocked with PBS containing 1% bovine serum albumin. To ascertain the cellular localization, cytoplasmic *vs* nuclear, for the p65 subunit of NF- κ B, cells were next incubated with a polyclonal rabbit anti-p65 immunoglobulin G (sc-372, Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer (1:1500) at 4°C overnight. After washing, Cy3-conjugated goat anti-rabbit Ig (Jackson Immuno Research Labs, West Grove, PA) in blocking buffer (1:1000) was added for one hour at room temperature. Cells were then imaged by confocal microscopy (Zeiss LSM 510, Carl Zeiss, Inc., Thornwood, NJ), as described previously in detail^[30].

Statistical analysis

All data represent at least three independent experiments and are expressed as the mean $\pm$ SE unless otherwise indicated. All of the data were expressed as means from three individual experiments. Differences between groups were determined by using the Student's *t*-test for unpaired observations.

RESULTS

Proteasome inhibition induces apoptosis selectively in human cholangiocarcinoma cells

MG132 was employed to inhibit proteasome proteolytic activity. Proteasome inhibition by MG132 induced apoptosis in all three human cholangiocarcinoma cells. Apoptosis was time-dependent and was maximal (65% $\pm$ 2%) at a

Figure 1 Morphologic assessment of MG132 induced death in KMCH, KMBC and Mz-ChA-1 cholangiocarcinoma and normal rat cholangiocytes (NRC) cell lines. **A:** MG132 induces apoptosis in all cholangiocarcinoma cell lines was maximal (> 90%) with a MG132 concentration of 10 ($\mu\text{mol/L}$); **B:** MG132 (1 $\mu\text{mol/L}$) induced apoptosis in the human KMCH cholangiocarcinoma cell line, but not in the nontransformed NRC cells; **C:** Apoptosis had the classic nuclear appearance as assessed by DAPI staining and fluorescence microscopy.

MG132 concentration of 1 $\mu\text{mol/L}$ at 24 h. Apoptosis was also concentration-dependent as maximal apoptosis (90%) was observed at 24 h in KMCH cells treated with 10 $\mu\text{mol/L}$ MG132 (Figure 1A, B, and C). In contrast, minimal cytotoxicity was observed in normal rat cholangiocytes treated with 10 $\mu\text{mol/L}$ MG132 (Figure 1B and C). As assessed by nuclear morphology, dead cells had the classic features of apoptosis (Figure 1C). We selected the KMCH cells for further mechanistic studies as this cell has been frequently employed for studies of human cholangiocarcinoma^[31].

Proteasome inhibition induces caspase-independent cell death

In many models, but not all, apoptosis is caspase-dependent. To determine if MG132 mediated apoptosis is caspase-

Figure 2 Caspase 3/7 activity and apoptosis in MG132 and staurosporine (SP) treated KMCH cells **A**: Cells were treated with MG132 1 $\mu\text{mol/L}$ and SP 5 $\mu\text{mol/L}$ for 16 h. Caspase 3/7 activity was increased SP cells ($P < 0.05$). In contrast, caspase activity was not different for MG132 treated cells vs controls. **B**: Cell death was assessed by morphologic criteria using DAPI and fluorescence microscopy. Cells were treated with SP or MG132 for 24 h in the absence or presence of the pancaspase inhibitor, QVD at 5 $\mu\text{mol/L}$. Note QVD blocked staurosporine, but not MG132, induced apoptosis. Data represent the mean $\pm$ SE of three separate studies.

Figure 3 Immunofluorescence for cell localization of the p65 NF- κ B subunit. Cellular compartmentation of the p65 NF- κ B subunit was examined in KMCH cells by confocal laser scanning microscopy. The p65 NF- κ B subunit was completely cytoplasmic in untreated KMCH cholangiocarcinoma cells; its localization also remained cytoplasmic following exposure to MG132 (1 $\mu\text{mol/L}$) for 24 h.

dependent in KMCH cells, we initially measured caspase activity (Figure 2A). Unexpectedly, an increase in caspase 3/7 activity was not observed in MG132 treated cells, although a 5-fold increase was readily observed in staurosporine treated cells, an agent which induces caspase-dependent cell death^[32]. Consistent with this observation, MG132 mediated apoptosis was also not inhibited by the pancaspase inhibitor QVD-OPh^[33]. In contrast, QVD-(0Ph) readily inhibited staurosporine mediated apoptosis (Figure 2B). Thus, in human cholangiocarcinoma cells, proteasome inhibition is associated with caspase- independent cell death.

NF- κ B is not constitutively activated in cholangiocarcinoma cells

Several effects of proteasome inhibitors including apoptosis seem to be mediated through inhibition of NF- κ B by blocking the degradation of I- κ B^[34]. Since this transcription factor is constitutively activated in many malignant cells, we examined NF- κ B activation in cholangiocarcinoma by immunocytochemistry^[35,36]. NF- κ B nuclear immunoreactivity was not identified in cholangiocarcinoma cells (Figure 3). Thus, NF- κ B is not constitutively activated in this cell type. Therefore, proteasome inhibition cannot induce apoptosis by blocking this pathway in this cell type.

Proteasome inhibition induces an ER stress pathway of apoptosis

To further examine the mechanisms of cell by MG132 in

these cells, ultrastructural studies of the MG132 treated cells were performed. Both in MG132 and bortezomib treated cells, prominent ER vacuolization was observed. This observation was extremely pronounced and readily apparent in all apoptotic cells (Figure 4A). Thus, proteasome inhibition is associated with morphologic evidence for ER dysfunction. The specificity of proteasome inhibition in this cell type was verified by examining mitochondrial function. The mitochondrial membrane potential is a sensitive indicator of mitochondrial function. In contrast to the ER structure and function alterations, proteasome inhibition by MG132 did not perturb the mitochondrial membrane potential. Indeed, integrated mitochondrial membrane fluorescence (average fluorescent intensity $\times$ pixels above background) was observed to be 168736 ± 17699 and 143269 ± 8859 in untreated and treated cells following 24 h of incubation, respectively ($P = \text{NS}$). Thus, proteasome inhibition by MG132 induces significant ER structural alterations in the absence of mitochondrial dysfunction.

Because the ER stress pathway of cell death can be associated with sustained increases in cytosolic-free Ca^{++} , we next measured cytosolic-free Ca^{++} in MG132 treated KMCH cells. Sustained, but very modest, increases in cytosolic-free Ca^{++} were observed after treatment with MG132 and bortezomib. Cytosolic-free Ca^{++} concentrations increased only 2 fold (129 ± 3 nmol/L to 244 ± 33 nmol/L with 1 $\mu\text{mol/L}$ MG132 and 292 ± 54 nmol/L with 1 $\mu\text{mol/L}$ bortezomib, $P < 0.05$). (Figure

Figure 4 A: Morphologic features of apoptosis induced by proteasome inhibitors, MG132 and bortezomib in KMCH cells. On the left up panel, transmission electron microscopic appearance of untreated cholangiocarcinoma cell is depicted (bar = 0.5 μm). In the right up panel, exposure to MG132 (1 μmol/L) for 24 h results in a distinctly dilated ER (arrowhead) (bar = 2 μm). On the bottom-left panel, similar results are observed with MG 132 (0.5 μmol/L) for 24 h (arrowhead) (bar = 2 μm). Note apoptotic morphology also characterized by condensation of heterochromatin; On the bottom-right panel, high magnification image of cytoplasm shows dilated ER (arrowhead) (bar = 2 μm); B: Ca²⁺ increases following treatment with proteasome inhibitors in KMCH cells. The time course of Ca²⁺ changes is demonstrated. At 24 h, Ca²⁺ levels were significantly increased in MG132 1 μmol/L and Bortezomib 1 μmol/L treated cells than untreated KMCH cells ($P < 0.05$); C: Intracellular Ca²⁺ chelation with BAPTA does not prevent apoptosis by MG132.

4B). Co-treatment of BAPTA-AM, a potent calcium-chelator, with MG132 did not reduce MG132 induced apoptosis (Figure 4C). Taken together, proteasome inhibition induces ER dysfunction and apoptosis in human cholangiocarcinoma cells. This ER dysfunction is characterized by morphologic changes in the ER and modest elevations in cytosolic-free calcium. However, the latter is unlikely to be the mediator of cell death.

Figure 5 The translational inhibitor cycloheximide inhibits MG132 induced cell death in KMCH cells. The translational inhibitor, cycloheximide (CHM) 50 μmol/L, completely inhibited MG132 1 μmol/L induced apoptosis in KMCH cells co-treated with both agents for 24 h. Cell death was assessed by morphologic criteria using DAPI and fluorescence microscopy. Data represent the mean ± SE of three separate studies.

Cycloheximide inhibits MG132 induced apoptosis

Proteasome inhibitors allow cellular protein synthesis to continue while they block degradation of damaged or unfolded proteins. Accumulation of unfolded proteins in the ER can trigger ER dysfunction and cell death. To ascertain if this is the mechanism of proteasome inhibitor mediated cell death in cholangiocarcinoma cells, the cells were co-incubated with MG132 plus cycloheximide, a protein synthesis inhibitor^[37]. Cycloheximide remarkably blocked MG132 induced apoptosis (7.5 vs 64.5%, $P < 0.05$) (Figure 5). This observation suggests active protein synthesis is necessary for the cytotoxicity of proteasome inhibition in human cholangiocarcinomas.

DISCUSSION

The principal findings of this study relate to the mechanism of cell death in human cholangiocarcinoma cells by proteasome inhibition. The results demonstrated that (1) proteasome inhibition induces apoptosis in cholangiocarcinoma cells; (2) the cell death is caspase-independent; and (3) is associated with morphologic changes of the ER; and (4) the cell death is dependent upon protein synthesis. The results support the further exploration of proteasome inhibition as a therapeutic strategy for human cholangiocarcinoma. The proteasome inhibitor MG132 induced time- and concentration-dependent cell death in all cholangiocarcinoma cell lines examined. These data suggest proteasome inhibitors are potential anti-cancer drugs for this neoplasm. This class of agents is currently employed for the treatment of multiple myeloma. Although, the efficacy of proteasome inhibitors for the treatment of solid tumors has been disappointing, this may not be necessarily true for cholangiocarcinoma. Despite the fact that cholangiocarcinoma is an epithelial cell tumor, it has many phenotypic features similar to multiple myeloma including interleukin 6 dependent survival signaling pathways^[38]. From this prospective, proteasome inhibitors may be anti-cancer drugs for cholangiocarcinoma as well.

Proteasome inhibitors have been reported to trigger caspase-dependent apoptosis by caspase-3 and 8 activation in multiple myeloma and chronic lymphocytic leukemia cells^[16,39]. The caspase dependent apoptosis can be induced by preventing proteasome degradation of Bim, a potent proapoptotic member of the Bcl-2 family of proteins. Bim accumulation then triggers the mitochondrial pathway of

apoptosis. Nevertheless, MG132 induced apoptosis was found to be caspase independent in cholangiocarcinoma cell lines as an increase of caspase-3/7 activity was not observed in our studies. Likewise, there was no inhibition of apoptosis by a pancaspase inhibitor. Consistent with the lack of caspase activity, the mitochondrial membrane potential was also unperturbed in treated cells. This observation suggests proteasome inhibition triggers an alternative cell death pathway in these cells. Proteasome inhibitors have several anti-tumoral mechanisms. One of them is by inactivation of the transcription factor, NF- κ B. Indeed, inhibition of NF- κ B is a prominent mechanism by which proteasome inhibition induces apoptosis. In response to cellular stress; I- κ B is degraded by proteasomes and NF- κ B released activating transcription of genes for several factors including apoptosis inhibitors^[16]. However, our current study demonstrated that NF- κ B is not constitutively active in KMCH, KMBC and Mz-ChA-1 cholangiocarcinoma cell lines and, therefore, proteasome inhibitors can not induce cell death in these cells by blocking NF- κ B activation.

The ER can be involved as a primary target organelle in apoptosis. Secretory proteins or proteins for plasma membrane are modified and acquire their correct folding conformation in the ER. Proteins that are unable to fold properly in the ER are degraded by the proteasome. It is proposed that proteasome inhibition can cause the accumulation of misfolded proteins, resulting in excessive ER stress^[17]. Transformed cells are particularly sensitive to the ER cell death pathway^[40]. Indeed, our ultrastructural studies demonstrated marked alterations of ER morphology by proteasome inhibition. Similar morphologic findings have also been reported in glioma cells undergoing apoptosis after treatment with proteasome inhibitor, bortezomib^[41]. The protein synthesis inhibitor cycloheximide did reduce cell death by the proteasome inhibitor MG132. This observation suggests that accumulation of misfolded or unfolded proteins following protein inhibition likely contributes to the ER morphologic changes and cell death observed in the current study. The ability of MG132 to induce cell death in the cells by a selective mechanism is of therapeutic interest. Drugs such as bortezomib warrant further attention as therapeutic agents in the treatment of cholangiocarcinoma.

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