Aryl Hydrocarbon Receptor Modulation of Tumor Necrosis Factor-α-induced Apoptosis and Lysosomal Disruption in a Hepatoma Model That Is Caspase-8-independent

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Recent studies suggest that the aryl hydrocarbon receptor (AhR) modulates susceptibilities to some pro-apoptotic agents. AhR-containing murine hepatoma 1c1c7 cultures underwent apoptosis following exposure to tumor necrosis factor-α (TNFα) + cycloheximide (CHX). In contrast, Tc9 cells, an AhR-deficient variant of the 1c1c7 line, were refractory to this treatment. AhR sense/antisense transfection studies demonstrated that AhR contents influenced susceptibility to the pro-apoptotic effects of TNFα + CHX. 1c1c7 cells and all variants expressed comparable amounts of TNF receptor-1 and TRADD. However, no cell line expressed FADD, and consequently pro-caspase-8 was not activated. AhR content did not influence JNK and NF-kB activation. However, Bid and pro-caspase-9, -3, and -12 processing occurred only in AhR-containing cells. Analyses of cathepsin B and D activities in digitonin-permeabilized cultures and the monitoring of cathepsin B/D co-localization with Lamp-1 indicated that TNFα + CHX disrupted late endosomes/lysosomes in only AhR-containing cells. Stabilization of acidic organelles with 3-O-methylsphingomyelin inhibited TNFα + CHX-induced apoptosis. The cathepsin D inhibitor pepstatin A suppressed in vitro cleavage of Bid by 1c1c7 lysosomal extracts. It also delayed the induction of apoptosis and partially prevented Bid cleavage and the activation of pro-caspases-3/7 in cultures treated with TNFα + CHX. Similar suppressive effects occurred in cultures transfected with murine Bid antisense oligonucleotides. These studies showed that in cells where pro-caspase-8 is not activated, TNFα + CHX can initiate apoptosis through lysosomal disruption. Released proteases such as cathepsin D trigger the apoptotic program by activating Bid. Furthermore, in the absence of exogenous ligand, the AhR modulates lysosomal disruption/permeability.

The late endosomal/lysosomal network (referred to hereafter as “lysosomes”) consists of a series of dynamic and interactive acidic organelles. Lysosomes are involved in numerous intracellular processes, including plasma membrane and receptor recycling, cholesterol trafficking, antigen processing, autophagy, sphingolipid metabolism, and protein degradation (1–3). Recent studies have also implicated roles for lysosomal proteases in the induction of apoptosis (reviewed in Ref. 4). Specifically, many exogenous and endogenous agents facilitate lysosomal membrane permeabilization, causing release of resident proteases into the cytosol and induction of apoptosis. Among such agents are hydrogen peroxide (5, 6), generators of reactive oxygen species (7–9), sphingosine (10), amyloid β-peptide (11), α-tocopheryl succinate (12), methylmercury (6), VP-16 (13), and tumor necrosis factor-α (TNFα) (14–19). Studies employing molecular and pharmacological approaches to regulate the expressions or the activities of the lysosomal proteases cathepsins B and D (8, 13–18), or employing cell lines having acidic organelles deficient in these proteases because of a trafficking disorder (20, 21), have clearly implicated a role for these two proteases in the pro-apoptotic effects of several of the above agents.

The mechanism by which lysosomal proteases induce apoptosis is speculative. Papers have appeared suggesting that lysates of partially purified lysosomes are capable of directly activating pro-caspase-3 (22, 23). However, recombinant cathepsins have failed to catalyze such a cleavage (24). Alternatively, several groups have demonstrated the in vitro cleavage of Bid by extracts from highly purified preparations of lysosomes (11, 21, 25) and by individual recombinant-derived cathepsins (24, 25). Several agents that induce lysosomal damage lead to Bid cleavage in vivo, and do so prior to the activation of the apoptosis (11, 18, 25, 26). Given the ability of Bid, the cleavage product of Bid, to induce cytochrome c release, and the role of the latter in the activation of the apoptosis, Bid cleavage represents a viable mechanism by which lysosomal disruption could induce apoptosis. Indeed, in the case of TNFα, the pro-apoptotic effects of the cytokine are muted in cells treated with small molecule inhibitors of Bid (27) or having reduced Bid contents (19). However, there is a confounder in most TNFα studies. Specifically, the cytokine generally activates pro-caspase-8, and caspase-8 also processes Bid to tBid. Hence, in principle, Bid could potentially be activated by TNFα exposure via multiple routes.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor. Over the past 3 decades the ligand-activated AhR has been documented to both positively and negatively regulate a variety of genes having dioxin-response elements in their promoters (28–31). The products of such genes are involved in diverse functions such as phase I and II metabolism, cell cycle regulation, apoptosis, and development.
AhR Modulation of TNF-induced Apoptosis

(28, 29, 32). In addition, recent studies suggest that the AhR may have ligand-independent activities. Specifically, homologs of the mammalian AhR receptor lacking a ligand-binding domain have been implicated in the developmental regulation of lower eukaryotes (33, 34). In addition, analyses of AhR-deficient cell lines suggest that AhR content, in the absence of exogenous ligands, regulates cell morphology (35), progression through G1 (35, 36), and susceptibility to the pro-apoptotic effects of ceramide (37), Fas ligand, CD95 cross-linking antibody (38), and the lysosomal photosensitizer N-aspartyl chlorin e6 (NPe6) (25).

To date, only a handful of proteins have been implicated as regulators of lysosomal fragility (15, 39–41). One such protein may be the AhR. Specifically, we have demonstrated that the AhR-containing murine hepatoma cell line 1c1c7 undergoes apoptosis in photodynamic therapy (PDT) protocols employing the lysosomal sensitizers NPe6 (9, 25). In PDT protocols, light-activated photosensitizers generate a pulse of singlet oxygen in the immediate vicinity of the sensitizer. In the case of 1c1c7 cells, NPe6 is exclusively sequestered in lysosomes (9). Irradiation of NPe6-loaded cells causes lysosomal disruption and the chronologically ordered cleavage of Bid, release of cytochrome c, and activation of the apoptosome (9). In contrast, although variants of the 1c1c7 line deficient in the AhR accumulated comparable levels of sensitizer in their acidic organelles, the latter were markedly resistant to lysosomal disruption following irradiation (25). Because restoration of AhR expression restored susceptibility to PDT-induced acidic organelle disruption and apoptosis, it appeared that AhR content regulated lysosome fragility (25).

In this study, the effects of AhR content on TNFα-induced apoptosis were investigated in cells of the 1c1c7 lineage. In such cells AhR content regulated susceptibilities to both TNFα-induced apoptosis and acidic organelle disruption. Knock-down of cellular Bid contents with Bid antisense oligonucleotides demonstrated that the BH3 protein mediated a portion of the pro-apoptotic effects of TNFα. Most unexpectedly, pro-caspase-8 was not activated by TNFα because of the absence of the adapter protein FADD and did not contribute to Bid activation. Instead, lysosomally derived cathepsin D was partially responsible for Bid cleavage and a portion of the apoptotic program induced by TNFα. Lysosomal protease release also led to an activation of pro-caspase-12. Hence, the 1c1c7 model proved to be a unique system for investigating the contributions of non-caspase-8 pathways to TNFα-mediated Bid cleavage and apoptosis, and for identifying the AhR as a putative modifier of lysosomal permeability.

EXPERIMENTAL PROCEDURES

Materials—The fluorescent molecule HO33342 was purchased from Molecular Probes (Eugene, OR). Ac-DEVD-AMC was obtained from BD Biosciences. AMC, GW4869, pepstatin A, cathepsin D substrate MOCac-GKPILFRKL(2,4-dinitrophenol)-D-R-NH2, cathepsin B substrate Z-RR-AMC, and recombinant human TNFα were purchased from Calbiochem. Bicinchoninic acid, leupeptin, and cycloheximide were purchased from BioMol (Plymouth Meeting, PA). Cathepsin D inhibitors diazo-acetyl-DL-2-aminohexanoic acid-methyl ester and acetyl pepstatin A from BioMol (Plymouth Meeting, PA). Cathepsin D inhibitors diazo-acetyl-DL-2-aminohexanoic acid-methyl ester and acetyl pepstatin A were obtained from Bachem (King of Prussia, PA). CA-074, CA-074-Me, E-64, and E-64d were obtained from Peptide Institute, Inc. (Louisville, KY). Z-Fa-fmk was obtained from Enzyme Systems Products (Livermore, CA). Recombinant murine Bid was from R&D Systems, Inc. (Minneapolis, MN). Lipofectin was purchased from Invitrogen. HA14-1 was obtained from Ryan Scientific, Inc. (Isle of Palms, SC).

Cell Culture and Viability Measurements—Murine hepatoma 1c1c7, Tao, TCVM, TAHr, WCMV, and WARV cell lines were obtained from J. Whitlock, Jr. (Stanford University, Palo Alto, CA). The origins and characterizations of these cell lines have been described (Ref. 37 and references therein). All cell lines were cultured at 37 °C in α-minimal essential medium (αMEM) containing 5% fetal bovine serum and 100 µg/ml streptomycin and 100 units/ml penicillin. The TCMV, TAHr, WARV, and WCMV lines were maintained in medium that also contained 500 µg/ml gentamicin.

Viability was assessed by counting cells with a hemocytometer, and scoring both morphology and trypan blue permeability. In the TNFα and TNFα + CHX treatment groups, a small percentage of cells expressed apoptotic morphological features (i.e. shrunken, blebbled), at the time of analyses, without being trypan blue-permeable. These cells, if allowed additional time, eventually became trypan blue-permeable. Hence, they were scored as being nonviable.

DEVDase Assay—Activations of procaspase-3 and -7 were analyzed by monitoring the generation of AMC from the caspase substrate Ac-DEVD-AMC. Changes in fluorescence over time were converted into picomoles of caspase activity by comparison to a standard curve made with AMC. DEVDase-specific activities are reported as nanomoles of product per min per mg of protein. The bicinchoninic acid assay, using bovine serum albumin as a standard, was used to estimate protein concentrations.

Western Blot Analyses—The reagents and procedures used for the preparation and processing of cell lysates, SDS-gel electrophoresis, and immunodetection of caspases-9 and -3 have been described in detail (25). Caspase-12 was detected in a similar manner using an affinity-purified rabbit polyclonal antibody raised against a keyhole limpet hemocyanin-coupled synthetic peptide of murine caspase-12 as the primary antibody (Cell Signaling Technology, Inc., Beverly, MA). The pro- and processed forms of caspase-8 were detected with either a rabbit polyclonal antibody raised to a recombinant protein corresponding to amino acids 217–350 of human caspase-8 (Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit polyclonal antibody raised to a synthetic peptide comprising amino acids 2–20 of human caspase-8 (Pharmingen).

Immunofluorescence Detection of Lamp-1 and Cathepsins B and D—Cultures grown on poly-L-lysine-coated coverslips were washed three times with PBS and fixed in 4% paraformaldehyde/PBS solution for 30 min. Thereafter, the fixed cells were washed three times with PBS and permeabilized with methanol for 5 min on ice. After washing (three times with PBS), nonspecific autofluorescence was quenched with 100 mM glycine/PBS for 30 min. Subsequent washes were carried out three times with PBS, 0.1% saponin. The coverslips were washed and then incubated with 500 nM HO33342/PBS. After a final wash, the coverslips were washed and then incubated with 5% goat serum albumin, PBS, 0.1% saponin for 1 h at 37 °C to block nonspecific immunoglobulin binding. The coverslips were washed and incubated with a 1:20 dilution of rabbit anti-human cathepsin D antibody (Oncogene Research Products, San Diego), or a 1:500 dilution of rabbit anti-mouse cathepsin B antibody (gift of Dr. B. Sloane, Wayne State University School of Medicine), and a 1:1000 dilution of the 1D4B rat anti-mouse Lamp-1 antibody (Development Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City) in blocking buffer for 2 h at 37 °C. After washing, secondary detection consisted of incubation with 1:200 dilutions of AlexaFluor 488 goat-anti-rabbit IgG and AlexaFluor 546 goat-anti-rat IgG (Molecular Probes) in blocking buffer for 1 h at 37 °C. The coverslips were washed and incubated for 5 min at room temperature with 500 nM HO33342/PBS. After a final wash, the coverslips were inverted onto a drop of SlowFade solution (Molecular Probes) on glass slides and sealed with acrylic nail polish. Digital images were captured using an Axiosplan 2 Imaging Microscope equipped with an ApoTome optical sectioning device (Carl Zeiss AG, Germany).
AhR Modulation of TNF-induced Apoptosis

Quantitative analyses of fluorescent images were performed with MetaMorph software (version 6.3r2, Molecular Devices, Sunnyvale, CA). Within each experiment, time settings for every exposure were held constant, and contrast/brightness settings were unaltered. The images captured by Axiovision were exported as TIFF format and then imported into MetaMorph as a dual channel image (i.e. channel A = red = Lamp-1 staining; channel B = green = cathepsin B/D staining). Individual cells were digitally isolated, and the inclusive threshold value was set at 50. Area measurements were used for co-localization analysis, which are expressed as percentage area of channel A above threshold that does not overlap channel B and vice versa. This measurement is independent of fluorescence intensity. For relative intensity measurements, the integrated value for channel A was divided by the integrated value for channel B. The integrated value represents the sum of all gray scale values for every pixel that is above threshold within a selected region.

Digitonin Permeabilization— Cultures established in 35-mm dishes were fed with fresh medium 16–20 h prior to treatment. Cultures were treated with nothing, CHX, TNFα, or CHX + TNFα for 0.5, 1, 2 or 3 h at 37°C prior to being washed three times with release buffer (1 mM PIPES, pH 7.0, 80 μM L-cysteine in Hanks’ balanced salt solution). Washed cultures were incubated at 37°C with 1 ml of release buffer with or without 0.1% Triton X-100 or varied concentrations of digitonin. After a 30-min incubation, culture fluids were removed and centrifuged at 13,800 g for 30 min at 4°C. Supernatant fluids were removed and transferred to a new tube and stored on ice until used in enzymatic assays.

Lactate Dehydrogenase and Cathepsin Assays— Supernatant fluids isolated from nontreated, digitonin-treated, and Triton X-100-treated cultures were centrifuged at 13,800 g for 30 min at 4°C. Supernatant fluids were removed and transferred to a new tube and stored on ice. Lactate dehydrogenase activity was determined using a lactate dehydrogenase assay kit (Molecular Devices, Sunnyvale, CA) microplate reader at 340 nm. Cathepsin B or cathepsins B/L activities were determined as described (9). Oligonucleotide Transfection—1c1c7 cells were suspended in αMEM containing 5% fetal bovine serum (without antibiotics) and plated in 35-mm culture dishes. Approximately 20 h after plating, the cultures were washed three times with PBS, and the medium was replaced with 0.8 ml of αMEM. Cultures were transfected with 0.2 ml of αMEM containing 5 μg of Lipofectin and in some cases 100 pmol of either murine Bcl-antisense or scramble sense oligonucleotides. The Lipofectin/oligonucleotide mixtures were prepared according to the instructions provided by the manufacturer.

In vitro Bid Cleavage Assay— The procedures used for in vitro cleavage of recombinant Bid by lysosomal extracts, and Western blot analyses of Bid cleavage products, have been described in detail (25).

RESULTS

Optimization of TNFα/CHX-induced Apoptosis— Exposure of 1c1c7 cultures to 30 pg/ml TNFα resulted in an ~10-fold increase in DEV-Dase within 4–8 h of treatment (Fig. 1A) and a small decrease in cell viability (Fig. 1C). Increasing the concentration of TNFα did not increase the proportion of dying cells or further elevate DEV-Dase activ-
AhR Modulation of TNF-induced Apoptosis

TNFα + CHX-induced Lysosome Disruption in 1c1c7 Cultures—Several studies have implicated a role for lysosomal proteases in TNFα-induced apoptosis (14–19). Preliminary studies using acridine orange to monitor lysosomal membrane integrity indicated that TNFα + CHX co-treatment affected these organelles in 1c1c7 cultures. Although acridine orange is commonly used to monitor acidic organelle intactness, it has limitations. Specifically, its fluorescence is pH-dependent. Conditions that cause acid organelle alklyation without membrane disruption cannot be distinguished from conditions that cause membrane permeability. In order to circumvent this caveat, we monitored the co-localization of the late endosomal/lysosomal luminal proteases cathepsins B and D with the late endosomal/lysosomal integral membrane protein Lamp-1 (1). Figs. 2 and 3 are representative of the effects of TNFα, CHX, and TNFα + CHX in 1c1c7 cultures on cathepsin B and D fluorescence staining patterns, respectively. Lamp-1 and cathepsins B and D exhibited punctate staining in both nontreated 1c1c7 cultures (Figs. 2, A and B, and 3, A and B). Both cathepsins extensively co-localized with Lamp-1 in nontreated 1c1c7 cultures (Figs. 2C and 3C). A visual inspection of the panels presented in Figs. 2 and 3 suggest that CHX or TNFα treatments either have no, or only subtle, effects on Lamp-1 and cathepsin B/D staining and Lamp-1/cathepsin co-localization. However, co-treatment of 1c1c7 cultures with CHX + TNFα markedly reduced cathepsin B (Fig. 2M) and cathepsin D (Fig. 3M) staining, without having perceivable affects on Lamp-1 staining (Figs. 2N and 3N). Indeed, the predominant red coloration of the cathepsin B/D + Lamp-1 overlay emphasizes the preferential losses of cathepsins occurring in TNFα + CHX-treated cultures (Figs. 2O and 3O).

MetaMorph analyses of individual cells were used to quantify the effects of CHX and TNFα treatments on Lamp-1 and cathepsin staining and co-localization in 1c1c7 cultures (Table 1). Three parameters were measured. First, the accumulative intensities of Lamp-1 and cathepsin B/D spots in individual cells were determined and expressed as the ratio of Lamp-1 to cathepsin staining. An increase in this ratio could reflect either an overall increase in Lamp-1 staining and/or losses of cathepsin staining. Second, an estimate was made of how many Lamp-1 (+) spots did not co-localize with cathepsin (+) spots. An increase in the percentage of Lamp-1 (+) spots having this phenotype, coupled with an increase in the Lamp-1/cathepsin intensity ratio, would be indicative of losses of co-localization because of the release of cathepsins from Lamp-1 (+) vesicles. Third, an estimate was made of how many cathepsin (+) spots did not co-localize with Lamp-1. Presumably, such a population is nonendosomal/lysosomal in nature. It should not be affected if our treatments exclusively target endosomes or lysosomes.

MetaMorph analyses of 1c1c7 cultures indicated that TNFα + CHX co-treatment dramatically increased both the Lamp-1/cathepsin D intensity ratio and the % of Lamp-1 (+) spots not containing cathepsin D (Table 1). Similar results, although of lesser magnitude, occurred with cathepsin B. TNFα + CHX co-treatment of 1c1c7 cultures neither increased nor decreased the % of cathepsin (+) spots that did not contain Lamp-1 (Table 1). Collectively, these findings suggest that lysosomal/endosomal cathepsin D, and to a lesser degree B, are released into the cytosols of 1c1c7 cells following TNFα + CHX co-treatment.

3-O-MeSM Regulation of Lysosome Fragility—3-O-Methylsphingomyelin (3-O-MeSM) is a synthetic analog of sphingomyelin in which the C-3 hydroxyl group has been replaced with a methoxy group. We recently reported that 3-O-MeSM is rapidly incorporated into the acidic organelles of 1c1c7 cells and prevents the rupture of lysosomes occurring in photodynamic therapy protocols employing the lysosomal pho-
tosensitizer NP6 (45). Strong protection was afforded by 50 μM 3-O-MeSM in the PDT protocol (45). Pretreatment with 50 μM 3-O-MeSM protected 1c1c7 cultures against the pro-apoptotic effects of TNFα + CHX co-treatment, as assessed by morphology (Fig. 4A), DEVDase activation (Fig. 4B), measurements of trypan blue permeability (Fig. 4C), and pro-caspases-3, -9, and -12 processing (Fig. 4D). Treatment of 1c1c7 cultures with 50 μM 3-O-MeSM did not alter cathepsin B (Fig. 2D), cathepsin D (Fig. 3D), or Lamp-1 (Figs. 2E and 3E) staining or cathepsin co-localization with Lamp-1 (Figs. 2F and 3F and Table 1). However, pretreatment with 3-O-MeSM prevented TNFα + CHX-mediated disruption of lysosomes. Specifically, cathepsins B and D remained co-localized with Lamp-1 in 1c1c7 cultures that had been treated with 3-O-MeSM prior to TNFα + CHX exposure (Figs. 2R and 3R and Table 1).

3-O-MeSM was originally described in the literature as a weak inhibitor of neutral sphingomyelinase (SMase) (46). Because the neutral SMase inhibitor GW4869 has been reported to suppress TNFα-induced apoptosis in MCF-7 cells (47), we thought it prudent to determine whether the effects of 3-O-MeSM in our system were related to neutral SMase. To examine this possibility, 1c1c7 cultures were pretreated with GW4869 prior to co-treatment with TNFα + CHX. Pre- and co-treatment with concentrations of GW4869 approaching its solubility limit in our culture medium (~6 μM) offered no protection against the pro-apoptotic effects of TNFα + CHX.

Lysosomal Protease Release Is Not a General Consequence of Apoptosis—The agent HA14-1 activates the intrinsic apoptotic pathway via disruption of the mitochondrial respiratory chain (48) and neutralization of the anti-apoptotic functions of Bcl-2/Bcl-XL (49). Exposure of 1c1c7 cultures to 15–25 μM HA14-1 induces apoptosis within 1 h (25, 45). Very recent studies suggest that this apoptotic response is unrelated to endosomal/lysosomal damage (45). Supplemental Fig. 1 depicts representative panels of Lamp-1 and cathepsin D staining in 1c1c7 cultures harvested 15, 30, and 60 min after exposure to 20 μM HA14-1. Meta-Morph analyses of individual cells indicated that HA14-1 treatment, unlike TNFα + CHX treatment, neither increased the relative intensity of Lamp-1/cathepsin D staining nor increased the % of Lamp-1 (+)
AhR Modulation of TNF-induced Apoptosis

Recent studies suggest that Bid is a mediator of the pro-apoptotic effects of TNFα (18, 19). To assess the contribution of Bid to the apoptotic program induced in our model by TNFα + CHX, 1c1c7 cultures were treated with either Bid antisense or scramble sense oligonucleotides. The Bid antisense oligonucleotide reduced Bid protein contents by >85% within 24 – 48 h of transfection (Fig. 5A). Bid contents began to recover thereafter but were still reduced 72 h after transfection by ~50%. In contrast, Bid protein contents were not affected by transfection of scramble sense oligonucleotides or by Lipofectin treatment (Fig. 5A).

Shrunken cells with apoptotic blebs were evident within 4.5 h of TNFα + CHX treatment in cultures that had been pretreated 48 h earlier with scramble sense oligonucleotides or Lipofectin (Fig. 5C). In contrast, no or few apoptotic cells were observed in cultures having reduced Bid levels during the same time period, or even 4 h later (Fig. 5C). DEVDase activities in the various treatment groups correlated with the morphological data (Fig. 5B). The kinetics of DEVDase activation following TNFα + CHX exposure, as well as actual activities, were identical for scramble sense-transfected and Lipofectin-treated 1c1c7 cultures on each of the examined days. In contrast, the kinetics of DEVDase activation were delayed in cultures having reduced Bid levels. Furthermore, DEVDase specific activities were lower in cultures having markedly reduced Bid contents (24 and 48 h post-transfection cultures).

Role of Caspase-3 in TNFα-mediated Bid Cleavage and Apoptosis—Bid activation via proteolytic cleavage can be catalyzed by caspase-8 or lysosomal proteases (24). Bid cleavage occurred in a time-dependent manner in 1c1c7 cultures co-treated with TNFα + CHX (Fig. 6A). Extracts of 1c1c7 lysosomes cleaved Bid in an in vitro assay (Fig. 6B). Bid cleavage was not affected by supplementing extracts with the caspase B inhibitor CA-074, the broad spectrum cysteine protease inhibitor E-64, the caspase B and L inhibitor Z-FA-fmk, or the serine protease inhibitor leupeptin. However, cleavage activity was lost in extracts pretreated with the caspase D inhibitor pepstatin A (Fig. 6B).

Pretreatment of 1c1c7 cultures with acetyl pepstatin A suppressed TNFα + CHX-mediated DEVDase activation in a concentration-dependent (Fig. 6C) and time-dependent (Fig. 6D) fashion. Maximal inhibition occurred with 1 μM acetyl pepstatin A. Similar results were observed in 1c1c7 cultures pretreated with 100 – 400 μM of the caspase D inhibitor diazoacetyl-Val-Leu-2-aminohepoxane acid methyl ester (DAME)(Fig. 6, C and D). Light microscopy indicated that concentrations of DAME > 400 μM caused cell stress by itself and hence were not employed. The protection mediated by the two caspase D inhibitors was highly reproducible but partial. Optimal concentrations of the two inhibitors suppressed overall DEVDase activities by ~50% (n = 6 experiments) and markedly improved cell survival (Fig. 6F). Pretreatment of 1c1c7 cultures with 1 μM acetyl pepstatin A also partially suppressed Bid cleavage and procaspase-3 cleavage (Fig. 6A). In contrast to the effects of the caspase D inhibitors, pretreatment of 1c1c7 cultures with the cell-permeable protease inhibitors CA-074-Me, E-64d, and Z-FA-fmk

### Table 1

| Cell type | Treatment | Type of caspase | Relative intensity of Lamp-1 (−) spots not containing caspase | Relative intensity of Lamp-1 (+) spots not containing caspase
|-----------|-----------|----------------|---------------------------------------------------------------|---------------------------------------------------------------|
|           |           |                |                                                                 |                                                                 |
| 1c1c7     | NT        | B              | 1.38 ± 0.10                                                | 32.1 ± 0.10                                                   |
|           | T         | B              | 1.26 ± 0.12                                                | 32.6 ± 0.12                                                   |
|           | C         | B              | 1.18 ± 0.12                                                | 32.4 ± 0.12                                                   |
|           | C + T     | B              | 1.27 ± 0.15                                                | 32.5 ± 0.15                                                   |
|           | 3OM       | B              | 1.21 ± 0.15                                                | 32.7 ± 0.15                                                   |
|           | C + T + 3OM| B              | 1.28 ± 0.15                                                | 32.8 ± 0.15                                                   |
| 1c1c7     | NT        | D              | 1.47 ± 0.18                                                | 33.2 ± 0.18                                                   |
|           | T         | D              | 1.30 ± 0.12                                                | 33.3 ± 0.12                                                   |
|           | C         | D              | 1.24 ± 0.12                                                | 33.4 ± 0.12                                                   |
|           | C + T     | D              | 1.34 ± 0.19                                                | 33.5 ± 0.19                                                   |
|           | 3OM       | D              | 1.38 ± 0.16                                                | 33.6 ± 0.16                                                   |
|           | C + T + 3OM| D              | 1.35 ± 0.19                                                | 33.7 ± 0.19                                                   |
| Tao       | NT        | B              | 1.31 ± 0.12                                                | 32.3 ± 0.12                                                   |
|           | T         | B              | 1.23 ± 0.15                                                | 32.4 ± 0.15                                                   |
|           | C         | B              | 1.16 ± 0.12                                                | 32.6 ± 0.15                                                   |
|           | C + T     | B              | 1.25 ± 0.19                                                | 32.7 ± 0.19                                                   |
|           | 3OM       | B              | 1.28 ± 0.17                                                | 32.8 ± 0.17                                                   |
|           | C + T + 3OM| B              | 1.31 ± 0.19                                                | 32.9 ± 0.19                                                   |
| WCMV      | NT        | B              | 1.22 ± 0.12                                                | 32.0 ± 0.12                                                   |
|           | T         | B              | 1.25 ± 0.16                                                | 32.1 ± 0.13                                                   |
|           | C         | B              | 1.18 ± 0.12                                                | 32.2 ± 0.13                                                   |
|           | C + T     | B              | 1.28 ± 0.17                                                | 32.3 ± 0.17                                                   |
|           | 3OM       | B              | 1.31 ± 0.17                                                | 32.4 ± 0.17                                                   |
|           | C + T + 3OM| B              | 1.35 ± 0.18                                                | 32.5 ± 0.18                                                   |
| WARV      | NT        | D              | 1.24 ± 0.13                                                | 32.1 ± 0.13                                                   |
|           | T         | D              | 1.27 ± 0.15                                                | 32.2 ± 0.14                                                   |
|           | C         | D              | 1.20 ± 0.12                                                | 32.3 ± 0.14                                                   |
|           | C + T     | D              | 1.32 ± 0.18                                                | 32.4 ± 0.18                                                   |
|           | 3OM       | D              | 1.35 ± 0.18                                                | 32.5 ± 0.18                                                   |
|           | C + T + 3OM| D              | 1.37 ± 0.19                                                | 32.6 ± 0.19                                                   |

*Values are greater than the nontreated group, p < 0.01.
AhR Modulation of TNF-induced Apoptosis

AhR-dependent Modulation of Sensitivity to TNFa/CHX-induced Apoptosis—The Tao cell line was derived from 1c1c7 cells (Ref. 37 and references therein). It contains greatly reduced levels of AhR (Fig. 7A). Relative to the parental line, Tao cultures were refractory to the pro-apoptotic effects of TNFa± CHX (Fig. 8, A and B). Specifically, almost no activation of DEVDase occurred in Tao cultures following exposure to TNFa, and only marginal activation occurred in cultures co-treated with TNFa and CHX (Fig. 8A). Differences in DEVDase activities were mirrored by dramatic disparities in the activations of pro-caspases-9, -12, and -3 in the two cell lines (Fig. 8B). Although coordinate losses and appearances of the pro- and processed forms of caspases-9, -12, and -3 were detectable in 1c1c7 cultures within 4–8 h of TNFa + CHX co-treatment, similar processing did not occur in Tao cultures.

Transfection of Tao cells with an Ah sense expression vector and subsequent selection of genetin-resistant cells gave rise to the TAHR cell line (35), which has AhR contents similar to 1c1c7 cells (Fig. 7A). Increased AhR expression restored sensitivity to the pro-apoptotic effects of TNFa + CHX (Fig. 8, C and D). The magnitude and kinetics of DEVDase activation and pro-caspase-9 and -3 processing in TAHR cultures were comparable with what were observed in wild type 1c1c7 cells (Fig. 8, compare A with C and B with D). In contrast, AhR content was not affected by stable introduction of the empty expression plasmid into Tao cells (resulting line designated TCMV; see Fig. 7A). TCMV cultures responded to TNFa ± CHX in a fashion comparable with Tao cells (Fig. 8, compare A with C and B with D).

Transfection of 1c1c7 cells with an Ah antisense expression vector gave rise to a stable cell line (designated WARV) (35) having diminished AhR content (Fig. 7A). The WARV line, relative to either wild type 1c1c7 cells or its vector-only cognate partner (Fig. 7A, WCMV cell line), was notably resistant to the pro-apoptotic effects of TNFa + CHX, as monitored by analyses of either DEVDase activities or pro-caspase processing (Fig. 8, E and F).

The elevated DEVDase activities/enhanced pro-caspase-3 processing occurring in TNFa + CHX co-treated AhR-containing cell types correlated with decreases in viability (supplemental Table 1). Specifically, whereas trypan blue permeability in 1c1c7 and WCMV cultures following singular CHX or TNFa treatment was not markedly different from that measured in control cultures (4–6%), permeability increased to ~40–50 within 10–13 h of TNFa + CHX co-treatment. Similarly, the absence of pro-caspase activation in TNFa + CHX co-treated AhR-deficient Tao and WARV cell lines was paralleled by no changes in trypan blue permeability (supplemental Table 1).

Recent studies have established that TNFa triggers the recruitment of the adapter protein TRADD to TNFR1 and the formation of a signaling complex capable of activating NF-kB and JNKs (50, 51). Subsequently, components of the complex dissociate and re-associate with

![FIGURE 4. 3-O-MeSM suppression of TNFa + CHX induced apoptosis. Cultures of 1c1c7 cells were treated with 30 pg/ml TNFa, 1 μg/ml CHX, and/or varied concentrations of 3-O-MeSM. In co-treatment protocols, 3-O-MeSM and CHX were added 90 and 30 min, respectively, prior to TNFa. Times indicated in the panels represent hours after TNFa addition. A, 1c1c7 cultures were treated with TNFa, CHX, and/or 50 μM 3-O-MeSM and photographed 9.5, 8.5, and 8 h after 3-O-MeSM, CHX, and TNFa additions, respectively. B, cultures were treated with TNFa, CHX, and various concentrations of 3-O-MeSM prior to being harvested for analyses of DEVDase activities at different times. Data represent means ± S.D. of triplicate analyses. Similar results were obtained in two additional experiments. Symbols are as follows: no treatment (x), CHX ( ), 50 μM 3-O-MeSM ( ), TNFa + CHX ( ), CHX + TNFa + 40 μM ( ), 50 μM ( ) or 60 μM ( ) 3-O-MeSM. C, cultures were treated with nothing (x), TNFa ( ), CHX ( ), 3-O-MeSM ( ), or TNFa + CHX + 3-O-MeSM ( ) for 8–10 h prior to being harvested for analyses of viability. Data represent means ± S.D. of three plates. D, cultures were treated with TNFa, CHX, and 50 μM 3-O-MeSM prior to being harvested for analyses of pro-caspase-3, -9, and -12 processing by Western blot analyses, using 25 μg of protein per lane.](image)

**TABLE 2**

| Treatment | Elapsed time | Relative intensity of Lamp-1/cathepsin | Lamp-1 (+) spots not containing cathepsin D | Cathepsin (+) spots not containing Lamp-1 | n |
|-----------|--------------|----------------------------------------|------------------------------------------|----------------------------------------|---|
| None      | min          | %                                      | %                                        | %                                      | ---|
| Solvent   | 60           | 1.30 ± 0.47                            | 53.0 ± 12.5                              | 36.2 ± 11.4                            | 18 |
| HA14-1    | 15           | 1.36 ± 0.51                            | 50.6 ± 10.4                              | 28.1 ± 10.1                            | 19 |
| HA14-1    | 30           | 0.91 ± 0.42*                           | 39.2 ± 13.6                              | 43.0 ± 14.7                            | 17 |
| HA14-1    | 60           | 1.08 ± 0.35*                           | 46.6 ± 10.3                              | 34.2 ± 9.4                             | 20 |

* Values are less than nontreated group, p < 0.01.

suppressed neither DEVDase activation (Fig. 6E) nor cell killing (Fig. 6F) following TNFa + CHX addition.

1c1c7 cultures were treated with MeSO, or 20 μM HA14-1 for the indicated lengths of time before being processed for Lamp-1 and cathepsin D staining and co-localization. Data were generated by MetaMorph analyses of 12–20 cells, and represent means ± S.D.

1c1c7 cells or its vector-only cognate partner (Fig. 7A, WCMV cell line), was notably resistant to the pro-apoptotic effects of TNFa + CHX, as monitored by analyses of either DEVDase activities or pro-caspase processing (Fig. 8, E and F).

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AhR Modulation of TNF-induced Apoptosis

FADD, forming a complex that facilitates the recruitment, oligomerization, and activation of pro-caspase-8 (50, 51). The 1c1c7 cell line and all of its variants expressed comparable levels of pro-caspase-8, TNFR1, and TRADD (Fig. 7A). AhR deficiency did not suppress formation of the initial signaling complex. Specifically, ligand engagement of the TNFR1 activated JNKs and NF-κB in both 1c1c7 and Tao cultures (Fig. 7, B and C, respectively). Exposure to TNFα + CHX caused rapid but transient activations of JNK1/2 in the two cell lines (Fig. 7B). Although JNK activation in Tao cultures with TNFα + CHX was comparable with what occurred with anisomycin, a lesser response was noted with 1c1c7 cultures (Fig. 7B). TNFα + CHX exposure also caused the rapid proteolytic degradation of 14kDa and subsequent NF-κB activation, as assessed by EMSA, in both cell lines (Fig. 7C). TNFα + CHX treatment stimulated NF-κB activation above what occurred with TNFα alone. Similar to the trend observed for JNK, a slightly stronger NF-κB EMSA signal was consistently observed with Tao cells (Fig. 7C and two additional experiments).

Most unexpectedly, we were unable to detect either the disappearance of pro-caspase-8 or the appearance of processed caspase-8 in 1c1c7, TAHR, or WCMV cultures treated with pro-apoptotic concentrations of TNFα ± CHX (Fig. 8, B, D, and F). In addition, we were unable to either suppress the development of apoptosis by co-treatment with the caspase-8 inhibitor IETD-fmk or detect the generation of an activity capable of cleaving the caspase-8 substrate Ac-IETD-AFC in 1c1c7 cells. Additional analyses revealed that the 1c1c7 cell line and its
AhR Modulation of TNF-induced Apoptosis

AhR Regulation of Lysosome Disruption by TNFα + CHX—Recent studies suggest that the lysosomes of Tao cultures are resistant to disruption by the oxidants generated in an NPe6/PDT protocol (25). Figs. 9 and 10 are representative of the effects of TNFα, CHX, and TNFα + CHX in Tao cultures on cathepsin B/D fluorescence staining patterns, respectively. Like 1c1c7 cultures, Lamp-1 and cathepsins B and D exhibited punctate staining in nontreated Tao cultures (Figs. 9, A and B, and 10, A and B). Both cathepsins extensively co-localized with Lamp-1 in nontreated Tao cultures (Figs. 9C and 10C). CHX or TNFα treatments either had no or only subtle effects on Lamp-1 and cathepsin B/D staining and Lamp-1/cathepsin co-localization in Tao cultures (Figs. 9 and 10 and Table 1). In marked contrast to what occurred in 1c1c7 cultures, the intensities of cathepsin B (Fig. 9F) and cathepsin D (Fig. 10F), and their co-localization with Lamp-1 (Figs. 9L and 10L), were not significantly altered following co-treatment of Tao cultures with TNFα and CHX (Table 1).

In order to confirm that the effects noted in Tao cultures were AhR-related, Lamp-1 and cathepsin B/D staining intensities and co-localization were also performed in a second AhR-containing and -deficient cell pair. Supplemental Figs. 2–5 depict representative panels of WCMV and WARV cultures stained after 4 h of singular or combined TNFα + CHX treatment. MetaMorph analyses of images are summarized in Table 1. CHX + TNFα co-treatment of AhR containing WCMV cells increased the Lamp-1/cathepsin B and D intensity ratios and the % of Lamp-1 (+) spots not containing cathepsin B or D (Table 1). Like 1c1c7 cells, the % of Lamp-1 (+) spots not containing cathepsin D in WCMV cells was greater than the % of Lamp-1 (+) spots not containing cathepsin B (Table 1). Conversely, co-treatment of AhR-deficient WARV cultures with TNFα + CHX affected neither Lamp-1/cathepsin B and D intensity ratios nor the % of Lamp-1 (+) spots not containing cathepsins B or D (Table 1). Hence, AhR deficiency suppressed the release of endosomal/lysosomal cathepsins by TNFα + CHX co-treatment in two different cell pairs.

Cathepsin Release in Digitonin Permeabilized Cultures—As a complement to the immunofluorescence studies, we developed an additional assay to monitor the release of lysosomal proteases into the cytosol. We reasoned that released lysosomal proteases might be measurable in the extracellular fluids of cultures permeabilized with digitonin. Lactate dehydrogenase (LDH) was used as a cytosolic marker protein. Treatment of 1c1c7 and Tao cultures with 1–4 μM digitonin caused concentration-dependent releases of LDH (Fig. 11A), cathepsin B (Fig. 11B), and cathepsin D (Fig. 11C). Concentrations of digitonin >4 μM facilitated additional releases of LDH but had minimal effects on cathepsins B and D. Digitonin caused comparable releases of LDH in 1c1c7 and Tao cultures throughout the concentration range investigated. However, comparable effects were not seen for cathepsins B and D. Greater releases of both cathepsins were observed in digitonin-treated Tao cultures (Fig. 11, B and C). In addition, the organelles containing cathepsins B and D responded differentially to digitonin. Specifically, cathepsin D was preferentially released in both cell lines following digitonin treatment (Fig. 11, B and C). This effect was particularly evident at concentrations of digitonin ≥2 μM.

In order to assess the effects of TNFα, CHX, and TNFα + CHX on lysosomal integrity, we monitored the releases of LDH and cathepsins B and D in cultures permeabilized with 2 μM digitonin, over a 3-h time period. Two μM digitonin was chosen because it represented a submaximal releasing concentration for all three enzymatic activities and caused comparable LDH release in both 1c1c7 and Tao cells.

TNFα + CHX co-treatment of 1c1c7 cultures did not trigger the release of LDH over the 3-h exposure period (Fig. 12A). However, co-treatment did trigger releases of cathepsins B and D (Fig. 12, B and C). Increases were reproducibly detectable within an hour and statistically significant within 2 h of treatment. In contrast, TNFα alone did not stimulate the release of any of the three enzymes. Similarly, CHX alone did not trigger releases of LDH and cathepsin B throughout the time course. However, CHX alone did stimulate statistically significant releases of cathepsin D in 1c1c7 cultures that were less than that observed in co-treated cultures.
Co-treatment of Taocultures with TNFα/H9251/H11001/CHX did not trigger the release of LDH (Fig. 12D). Although small releases of cathepsin B (Fig. 12E) and cathepsin D (Fig. 12F) occurred in co-treated cultures, the measured activities were not statistically different from the zero time control. It should be noted that the zero time cathepsin values observed in these studies are a consequence of the digitonin concentration (2 μM) used to render the cells permeable (Fig. 11, B and C) and do not reflect actual basal cytosolic activities.

**DISCUSSION**

TNFα engagement of the TNFR stimulates pro-survival and/or pro-apoptotic pathways in several cell types (50, 51). In the case of the latter, the pro-apoptotic effects of TNFα are often attributed to its activation of pro-caspase-8. Caspase-8 can either activate pro-caspase-3 directly or indirectly via its cleavage of Bid to tBid and the eventual activation of pro-caspase-9. Although 1c1c7 cells contained pro-caspase-8, it did not undergo processing following exposure of cultures to TNFα/H9251/H11001/CHX. This deficiency probably reflects the observed absence of FADD in 1c1c7 cultures. Without FADD, pro-caspase-8 cannot be recruited to the activated TNFR and undergo the oligomerization and conformational changes necessary for activation. Hence, TNFα + CHX-induced apoptosis in 1c1c7 cultures is mediated by a non-caspase-8 pathway.

Several studies, including this one, demonstrate that TNFα activation leads to lysosomal permeabilization in cultured cells (14, 16, 18, 19). Molecular and pharmacological approaches have implicated several lysosomally derived cathepsins as mediators of the pro-apoptotic effects of TNFα (13–18). Indeed, in this study pretreatment with 3-O-MeSM prevented TNFα + CHX-mediated acidic organelle destabilization, protease release, and the subsequent induction of apoptosis. In principle, released proteases could contribute to non-caspase-8-mediated pro-caspase-9 activation.
apoptosis via several mechanisms. First, Ishisaka et al. (22, 23) reported an activation of pro-caspase-3 in cytosolic fractions upon incubation with lysosomal extracts. Although the identity of the activating enzyme is not known, in vitro cleavage assays have ruled out cathepsins as the species responsible for pro-caspase-3 activation (24). Second, inhibitor studies suggest that cathepsin D is capable of activating Bax (52). Such activation could facilitate cytochrome c release and activation of the apoptosome. Third, lysosomal extracts (9, 24–26) and some cathepsins (18, 24, 26) are capable of converting Bid to tBid. Bid cleavage has been documented in several cell types following disruption of lysosomes (this study and Refs. 18 and 26). Activation of Bid, and the induction of apoptosis, can be inhibited to variable degrees by co-treatment with cathepsin inhibitors (this study and Refs. 18 and 26). Finally, released lysosomal constituents may directly, or indirectly, damage or stress other organelles capable of triggering an apoptotic program. Incubation of isolated mitochondria with cathepsins B and D in vitro has been reported to provoke the generation of reactive oxygen species and the release of cytochrome c (53). Alternatively, released lysosomal proteases may target the endoplasmic reticulum and facilitate pro-caspase-12 activation. Indeed, in this study, TNFα + CHX co-treatment of 1c1c7 cultures activated pro-caspase-12. This activation, as well as the disruption of lysosomes, was suppressed by pretreatment with 3-O-MeSM. We recently reported that 3-O-MeSM suppresses the rupture of lysosomes in NPe6/PDT protocols but promotes or has no effects on the pro-apoptotic activities of HA14-1, staurosporine, tunicamycin, and thapsigargin in 1c1c7 cultures (45). Because the latter two agents cause endoplasmic reticulum stress and pro-caspase-12 activation (54–56), it seems likely that the protective effects of 3-O-MeSM in our studies are upstream of the endoplasmic reticulum. Although TNFα-mediated activation of pro-caspase-12 has been reported (57), this is the first study that we are aware to implicate a lysosomal factor in that process.

At least two lines of research indicate that Bid is an important mediator of the pro-apoptotic effects of TNFα. First, Werneburg et al. (19) reported that cultured fibroblasts deficient in Bid are more resistant than their Bid-containing counterparts to the pro-apoptotic effects of TNFα. Second, the pro-apoptotic effects of TNFα can be suppressed by co-incubation with small molecule inhibitors of Bid (27). In this study the pro-apoptotic effects of TNFα were both delayed and attenuated in 1c1c7 cultures having reduced Bid contents as a consequence of pretreatment with Bid antisense oligonucleotides. Bid depletion reduced DEVDase activation (a measure of caspase-3/7 activities) by ∼50% in TNFα + CHX-treated cultures. Comparable effects were also achieved by treating cultures with Bl-C69, a small molecule inhibitor of Bid (27). Hence, Bid plays a role in TNFα-induced apoptosis in 1c1c7 cultures. However, it cannot be the sole factor. Although others have reached a similar conclusion about Bid, in this study we have been able to obviate caspase-8 contributions to TNFα-induced Bid cleavage and apoptosis.

The extended loop of Bid, which contains the caspase-8 cleavage site, is cleaved in vitro by extracts of purified lysosomes (9, 24, 25) and by cathepsins D (18), B, H, K, L, and S (24, 26). Although there are conflicting reports on the ability of cathepsin D to cleave Bid in vitro (18, 26), the lack of cleavage noted in one study may be the consequence of performing the assay at pH 7.2 (26), which was subsequently reported to be
Nevertheless, studies by Heinrich et al. (18) and Bidere et al. (52) strongly suggest that cathepsin D, but not papain-like cysteine protease (cathepsins B, L, H & K), mediates a portion of TNFα-induced apoptosis and Bid cleavage in some cell types. Similarly, our studies show that pepstatin A was the only cathepsin inhibitor able to block lysosomally mediated Bid cleavage in an in vitro assay and suppress TNFα + CHX-induced apoptosis. It should be noted that other investigators have implicated cathepsin B in both Bid cleavage (24, 26) and TNFα-mediated apoptosis (14–16). The basis for the differences among studies is not known. It is conceivable that the cell types used in the various studies contain different ratios of the relevant cathepsins or have different cytoplasmic contents of cystatins or stefins. High levels of the two latter cysteine protease inhibitors could negate contributions by cathepsin B to Bid cleavage.

The pro-apoptotic effects of TNFα are often offset by its ability to activate NF-κB signaling and induce the expressions of proteins involved in cell survival (50, 51). The protein synthesis inhibitor CHX is commonly used to negate the pro-survival activities of TNFα. Indeed, co-treatment of 1c1c7 cultures with TNFα and CHX potentiates the pro-apoptotic response to TNFα, and this potentiation occurred at concentrations of CHX that suppressed protein translation. Although the effects of CHX are generally attributed to its suppression of the translation of pro-survival proteins such as cFLIP or cIAP, there may be a second mechanism whereby CHX amplifies the pro-apoptotic effects of TNFα. Specifically, both the cathepsin/Lamp-1 co-localization studies and the analyses of cathepsin activities in digitonin-permeabilized cultures suggested that TNFα + CHX treatment promoted the release of lysosomal proteases. When used singularly, neither agent promoted the release of cathepsins in the co-localization assay. Similarly, TNFα did not stimulate cathepsin release in the digitonin permeabilization assay. However, some release did occur in CHX-treated cultures. These findings emphasize that CHX many facilitate apoptosis in TNFα + CHX co-treatment protocols by mechanisms other than suppressing the synthesis of pro-survival proteins.

Previous analyses of 1c1c7 cells and its AhR-containing and -deficient variants indicated that AhR content differentially influences susceptibilities to subclasses of apoptotic inducers. Specifically, the 1c1c7, TAHR, and WCMV cell lines, and their respective AhR-deficient counterparts (Tao, TCMV, and WARV), exhibit comparable susceptibilities to the pro-apoptotic effects of HA14-1, staurosporine, and doxorubicin (25, 37). In contrast, the three AhR-deficient lines, relative to their AhR-containing counterparts, are less sensitive to the pro-apoptotic effects of C2-ceramide (37), NPe6/PDT (25), and TNFα + CHX (this study). It should be noted that our observations regarding AhR deficiency and susceptibility to pro-apoptotic agents are not unique. Park et al. (38)
AhR Modulation of TNF-induced Apoptosis

reported that FasL-mediated apoptosis in AhR-deficient rat BP8 hepatoma cells required AhR expression. Similarly, Ah null mice, unlike wild type mice, were refractory to the cytotoxic effects of infused CD95 cross-linking antibody (38).

A potential link between TNF + CHX and NPe6/PDT are their abilities to permeabilize lysosomes (this study and see Ref. 25). Recent studies indicate that C2-ceramide also triggers cathepsin release into the cytosols of 1c1c7 cells prior to the development of apoptosis.3 Furthermore, several studies have documented lysosomal permeability and a role for lysosomal proteases in CD95-mediated apoptosis (21, 58). Hence, the resistance of AhR-deficient cells to the pro-apoptotic effects of these agents may relate to either a property of their acidic organelles or their inability to activate processes causing lysosomal permeability. Although the current TNFα + CHX study does not distinguish between the two possibilities, Nilsson et al. (59) reported that lysosomes are susceptible to disruption by oxidants, and TNFα in some cell types generates significant levels of oxidants (60, 61). In the case of the NPe6/PDT protocol, the photosensitizer is sequestered in lysosomes (9). Analyses of AhR-containing and -deficient cells demonstrated that the acidic organelles of the latter were resistant to the oxidant damage caused by singlet oxygen generated within the organelles following photoactivation of NPe6 (25). Given that the oxidants were generated within the organelles, and the amounts of sensitizer in AhR-deficient cells were comparable with if not greater than their AhR-containing counterparts (25), it seems likely that AhR-deficiency affects properties of the lysosomes themselves (see below).

A handful of proteins may function as modifiers of lysosome permeability. Among such proteins are p53 (41), the phosphorylated form of Bcl-2 (15, 17), and Hsp70 (40). Although phosphatidyl form of Bcl-2 (39), cathepsin B (15, 17), and Hsp70 (40). Although cathepsin B and activated p53 have been implicated in facilitating the disruption of acidic organelles (15, 17, 41), phosphorylated Bcl-2 and Hsp70 have been assigned protective roles (39, 40). It is not known whether any of these proteins are related to how AhR content influences lysosomal fragility in our cell lines. Molecular and pharmacological approaches have implicated roles for SMases in TNFα-induced apoptosis (47, 62, 63). Ceramide, the product of SMases, is converted by ceramidases to sphingosine. At least two studies suggest that sphingosine functions as a lysosomal detergent (10, 14). 3-O-MeSM was originally described as a very weak neutral SMase inhibitor (46) and was effective in this study in suppressing both TNFα + CHX-induced lysosome permeability and apoptosis. Although the neutral SMase inhibitor GW4869 did not suppress TNFα + CHX-induced apoptosis in our model, we have not performed comparable studies with acidic SMase inhibitors. However, we think it is unlikely that SMases or sphingosines are responsible for lysosome permeability and the apoptosis occurring in our system. Specifically, we have been unable to detect acidic or neutral SMase activation in 1c1c7 cells following a 0.5–180-min exposure to TNF.3 Furthermore, although cathepsin B can be readily released from highly purified preparations of 1c1c7 lysosomes by in vitro exposure to Triton X-100, no release occurs following exposure to 1–10 μM sphingosine.3 Our studies suggest that lysosomal lipid composition may differ in AhR-containing and -deficient cell lines. Specifically, digitonin treatment released greater percentages of total cathepsin B and D activities in the AhR-deficient Tao line. Digitonin permeabilizes membranes by interacting with cholesterol. Filipin staining of our cell lines indicates that C2-ceramide also triggers cathepsin release release following exposure to Triton X-100, no release occurs following exposure to 1–10 μM sphingosine.3

characteristics of cell lines derived from patients suffering from the lysosomal storage disease mucolipidosis II (inclusion cell disease or I cell disease), including resistance to agents causing lysosomal breakage (20, 21). I cells, as well as many other lysosomal storage diseases, accumulate unesterified cholesterol in their lysosomes (64–66). Unesterified cholesterol and sphingomyelin largely determine membrane fluidity (67, 68). Increases in unesterified membrane cholesterol content are often paralleled by increases in sphingomyelin content (67). Increases in the two favor a liquid ordered state (67). Although speculative, lysosomal accumulation of unesterified cholesterol may alter susceptibility to agents causing lysosomal destabilization. Indeed, recent studies indicate that lysosomal incorporation of a sphingomyelin analog increases resistance to oxidant-induced lysosomal permeability (45).

In summary, analyses of the TNFα-induced apoptotic program in cells of the 1c1c7 lineage have yielded novel information about the role of lysosomal proteases in apoptosis and functions of the AhR. Specifically, it is unlikely that caspase-8 functions as an initiating caspase in 1c1c7 cells because they are FADD-deficient (Fig. 13). TNFα will initiate apoptosis in 1c1c7 cells if cultures are co-treated with CHX. This activity of CHX reflects minimally two effects. First, CHX may suppress the translation of mRNAs encoding survival proteins induced as a consequence of TNFα activation of the NF-κB pathway. Second, CHX + TNFα facilitates destabilization/permeability of lysosomes. This permeabilization does not occur in AhR-deficient cells or cultures pretreated with 3-O-MeSM (Fig. 13). Because AhR deficiency also prevents NPe6 disruption of lysosomes in PDT protocols (24), it appears that AhR content influences lysosome permeability. As such, this represents a new and novel function for the AhR. Released lysosomal factors can initiate apoptosis by cleaving Bid or activating pro-caspase-12 (Fig. 13). Once formed, caspase-12 can activate pro-caspase-9 directly (54, 55), whereas tBid stimulates cytochrome c release and apoptosis formation (69, 70). The 1c1c7 model represents a useful system for assessing the roles of lysosomal proteases in apoptosis and deciphering the factors affecting lysosomal fragility/permeability.

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