Essential role for cathepsin D in bleomycin-induced apoptosis of alveolar epithelial cells

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ANGIOTENSIN II (ANG II) is a potent inducer of apoptosis in alveolar epithelial cells (AEC) and is synthesized and released from AEC undergoing apoptosis in response to other stimuli (3). Work from this laboratory has shown that ANG II is secreted by AEC challenged in vitro with Fas ligand (15) or TNF-α (14) and demonstrated that the production of ANG II is required for the signaling of apoptosis by these inducers. More recent investigations showed that AEC apoptosis in response to the fibrogenic agent bleomycin (Bleo) requires the autocrine synthesis of ANG II and the subsequent binding of ANG II to receptor subtype AT1 (8). Apoptosis of AEC in response to the fibrogenic antiatherosclerotic agent amiodarone also is blocked by ANG receptor AT1-selective antagonists (3, 13). Together, these findings have led to the theory that autocrine production of ANG II by AEC de novo, i.e., from the precursor angiotensigen, is a common event required for AEC apoptosis regardless of the initiating stimulus (11).

Primary cultures of rat AEC were shown to constitutively express low but functional levels of angiotensin converting enzyme (ACE) mRNA (16) and to respond to ACE inhibitors such as captopril or lisinopril (12, 14). However, the identity of the enzyme(s) in AEC that act upstream of the ACE reaction, i.e., performing the conversion of angiotensigenin to ANG I (the substrate for ACE), remains unknown. In the serum, the conversion of liver-derived circulating angiotensigenin into ANG I is accomplished by the kidney-derived enzyme renin; this system is now viewed as the classical “endocrine” renin-ANG system (2). In contrast, tissue-specific “local” ANG systems exist in many tissues as either “extrinsic” systems (i.e., dependent on one or more components of the endocrine system) or as “intrinsic” ANG systems in which all the enzymes and substrates required for the production of ANG II are synthesized locally. In local intrinsic systems outside the lung, the primary asparl protease that converts newly synthesized angiotensigenin to ANG I is cathepsin D (CatD, Ref. 17), a ubiquitous lysosomal asparl protease expressed by virtually all cells (5). The identity of this asparl protease in the pulmonary local ANG system is the subject of this study.

A critical role for CatD in the execution of apoptosis has been shown previously in a variety of cell types including kidney cells lines (4), PC12 cells, and dorsal root ganglion-derived neurons (5) and in ML1 leukemia or U1752 lung cancer cells exposed to etoposide or adriamycin (18). In those studies, apoptosis in response chemical stimuli or trophic withdrawal could be prevented by the asparl protease inhibitor pepstatin A (pepA) or by antisense oligonucleotides against CatD mRNA. One investigation of CatD-dependent apoptosis in neuronal cells led to the hypothesis that activation of CatD by apoptosis inducers leads to the generation of an unidentified “bioactive molecule” that is required for the signaling of apoptosis but is either degraded or expressed at low levels in viable cells under basal unstimulated conditions (5).

In the light of previous demonstrations that AEC apoptosis requires the autocrine synthesis of ANG II and the documented ability of CatD to convert angiotensigenin to ANG I, we hypothesized that CatD might be required for AEC apoptosis. We also theorized that the primary function of CatD in AEC apoptosis is the conversion of angiotensigenin to ANG I. We report here that AEC apoptosis in response to Bleo is inhibited by CatD knockdown as a result of its blockade of ANG II synthesis.
MATERIALS AND METHODS

Reagents and materials. The ANG receptor AT1-selective antagonist L158809 was obtained from Merck (West Point, PA). The CatD fluorescent substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Arg-Leu-Lys(Dnp)-d-Arg-NH2 (19) was obtained from Peptides International (Louisville, KY). Bleo, anti-ANG antibodies, captopril, and saralasin were obtained from Sigma (St. Louis, MO). A kit for ELISA quantification of ANG II was obtained from Peninsula Laboratories (San Carlos, CA). All other materials were of reagent grade and were obtained from Sigma.

Cell culture. Primary AEC were isolated from adult male Wistar rats as described earlier (15). The primary cells were studied at day 2 of culture, a time at which they are type II cell-like by accepted morphologic and biochemical criteria (9). Primary cell preparations were of >90% purity assessed by acridine orange staining as described previously (14, 15). All cells were grown in 24- or 6-well chambers and were analyzed at subconfluent densities of 80–90%. All subsequent incubations with Bleo and/or other test agents were performed in serum-free medium. The cells were exposed to caspase inhibitors or antagonists of the ANG system 30 min before exposure to Bleo for 1–20 h as indicated.

Quantitation of nuclear fragmentation and caspase-3 activity. Detection of apoptotic cells with propidium iodide (PI) was conducted as described earlier (14, 15) following digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5 µg/ml PI. In these assays, detached cells were retained by centrifugation of the 24-well culture vessels during fixation with 70% ethanol. Cells with discrete nuclear fragments containing condensed chromatin were scored as apoptotic. As in earlier publications, the induction of apoptosis was verified by in situ end labeling of fragmented DNA (8). Apoptotic cells were scored over a minimum of four separate microscopic fields from each of at least three culture vessels per treatment group.

The enzymatic activity of caspase-3 was measured in adherent cells incubated for 20 h with the membrane-permeable substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Upstate Biotechnology, Saranac Lake, NY) at 50 µM final concentration. Quantitation of the fluorescent product was achieved with a Biotek FL600 fluorescence plate reader. Fluorescence values were normalized to cell number determined on the same culture well after cell fixxing and staining of DNA with PI (15).

Assay of CatD activity. The enzymatic activity of CatD was determined with the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Arg-Leu-Lys(Dnp)-d-Arg-NH2 as described by its inventors (19). Briefly, aliquots of AEC lysates or concentrated cell culture media were incubated in opaque 96-well culture plates (suitable for top reading in a fluorescence plate reader) in 1.0 M sodium acetate buffer, pH 4.0, containing 50 µM fluorogenic substrate. The total volume of reaction buffer, including sample, was 100 µl. In the case of cell lysates, equal amounts of lysate protein were assayed per culture vessel in triplicate. For concentrated cell culture media, the volume of medium assayed was normalized to equivalent amounts of cells used for conditioning the media, as determined by the lysate protein concentration. Initial rates of fluorescent product formation were obtained from the slope of continuous readings taken over 30 min following the addition of substrate. Initial reaction rates were linear with both time and protein concentration (see RESULTS).

RT-PCR and antisense experiments. Quantitative real-time RT-PCR was performed by standard protocols in the Genomics Technology Support Facility, Michigan State University. Primer sequences were designed on the basis of published sequence information and the public domain software Primer3 (Massachusetts Institute of Technology, Cambridge, MA). The annealing temperatures for PCR reactions were optimized for each primer by preliminary trials. The identity of expressed genes was determined by expected size of the PCR product in 1.6% agarose gels, followed by excision and sequencing of the PCR product.

For RT-PCR of rat CatD, the primers used were as follows: (primer set 1) coding = 5'-ACACTGTGTCGGTTCCATGT-3' and uncoding = 5'-TGGGATGATACGCTTC-AG-3', which produces a PCR product of 101 bp, and (primer set 2) coding = 5'-GGGTTC-TTGATCTGATCATCTC-3' and uncoding = 5'-TGGGACCTTTAAG-GATCA-GG-3', which produces a PCR product of 141 bp.

For antisense studies, phosphorothioated control and antisense oligonucleotides against CatD (22 mers) were designed through published sequence information and public domain software, synthesized, and transfected into primary rat AEC (both at 40 nM final concentration) using the lipofectin reagent Oligofectamine (Invitrogen Life Technologies, Grand Island, NY) at 4 µM/ml as the vehicle, diluted in the OPTIMEM medium accompanying the lipofectin. The control nucleotides were of the same length and base composition as the antisense but with scrambled sequence. The oligonucleotide-lipofectin ratio was optimized to yield transfection efficiencies of 50–75% with minimal cell loss or detachment. Transfection efficiency was monitored with FITC-labeled 25-mer oligonucleotide for luciferase (not shown). We conducted transfections for 4 h followed by washing them five times with serum-free cell culture medium, as described earlier (14, 15). Immediately thereafter, Bleo or vehicle was applied as indicated for 20 h. The transfection protocol itself had no significant effect on basal or Bleo-induced apoptosis (see RESULTS). Phosphorothioated oligonucleotide sequences were as follows: (CatD antisense) 5'-CATATAGTTTTGCTTCTGTCCT-3' and (CatD scramble) 5'-TGCCCTATATGTTAGTTC-TTTC-3'.

RESULTS

Measurements of CatD enzymatic activity with the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Arg-Leu-Lys(Dnp)-d-Arg-NH2 revealed CatD enzymatic activity in lysates of purified rat AECs (Fig. 1); the generation of fluorescent product was linear with time and lysate protein concentration. Incubation of primary AEC cultures with Bleo for 20 h, at a concentration previously shown to stimulate AEC apoptosis (25 nM/ml, Ref. 8), significantly increased the activity of CatD in both AEC lysates and in the serum-free cell culture medium (Fig. 2). The aspartyl protease inhibitor pePA inhibited the CatD activity by >90%.

Apoptosis inducers are known to increase CatD activity and mRNA in other cell types (5, 18). Analyses of CatD mRNA in primary AECs by RT-PCR (Fig. 3A) revealed PCR products of the correct size expected from two different primer sets but no

![Fig. 1. Cleavage of a fluorogenic substrate for cathepsin D (CatD) is dependent on time and protein concentration. Lysates of primary alveolar epithelial cells (AECs) were incubated with the fluorogenic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Arg-Leu-Lys(Dnp)-d-Arg-NH2, and generation of fluorescent product was monitored continuously over 30 min (see MATERIALS AND METHODS for details). Note linearity of product formation with time and amount of AEC lysate. mFU, milli-fluorescence units.](http://ajplung.physiology.org/10.1152/ajplung.00332.2004)
apparent change in response to Bleo. Sequencing of both PCR products verified the specificity of the PCR for rat CatD (not shown). Quantitative analyses of CatD mRNA by real-time PCR were unable to detect significant changes in the mRNA in response to Bleo challenge (Fig. 3B). In contrast, Western blotting of AEC lysates and culture media with CatD-specific antibodies and high-resolution gels (Fig. 4) revealed Bleo-induced increases in immunoreactive CatD proteins in the cell culture medium but apparently not in the cell lysates. Bleo increased isoforms of CatD of apparent molecular masses of 52, 48, and 44 kDa in the culture medium, whereas a 44-kDa protein was the major immunoreactive isoform of CatD present in AEC lysates.

To begin determining whether CatD might play a role in apoptosis of AECs as it does in other cell types, we evaluated AEC apoptosis in the presence and absence of the aspartyl protease inhibitor pepstatin A (pepA). In Fig. 5, pepA inhibited Bleo-induced nuclear fragmentation of primary AEC by 76% ($P < 0.01$) and reduced Bleo-stimulated caspase-3 activity by 47% ($P < 0.05$). The pepA alone did not affect basal nuclear fragmentation or caspase-3 activity.

As an alternate test of the role of CatD in AEC apoptosis, phosphorothioated antisense oligonucleotides specific for CatD mRNA were designed and transfected transiently into primary rat AEC with lipofectin (Fig. 6). In Fig. 6A, the antisense oligonucleotides significantly reduced the immunoreactive CatD released into AEC culture media, as determined by Western blotting on low-resolution gels. In contrast, scrambled-sequence control oligonucleotides, of the same length and base composition as the antisense, did not reduce CatD immunoreactivity. In Fig. 6B, pretreatment of primary AECs with the same antisense oligonucleotides used in Fig. 6A reduced Bleo-induced nuclear fragmentation by 48% ($P < 0.05$).

Recent work from this laboratory showed that the induction of AEC apoptosis by Bleo requires de novo synthesis of ANG II and its subsequent binding to ANG receptor subtype AT$_1$ (8). To begin addressing the theory that the primary role of CatD in AEC apoptosis is its ability to process angiotensinogen to the peptide ANG I, we evaluated CatD knockdown strategies for the ability to prevent AEC apoptosis in response to a synthetic angiotensinogen fragment consisting of amino acids 1–14. This domain of angiotensinogen contains the catalytic activity of CatD for processing Ang I.

Fig. 2. Bleomycin (Bleo) upregulates CatD activity and release from AECs. Primary cultures of rat AECs were exposed to Bleo for 20 h at a concentration known to induce AEC apoptosis (25 mU/ml, Ref. 8). CatD activity was measured in cell lysates as described in Fig. 1, in the presence or absence of the aspartyl protease inhibitor pepstatin A (pepA). Inset: CatD activity was measured in concentrated cell culture medium collected from Bleo-treated (B1) and untreated (C) cells studied in A. Bars are means ± SE of $n = 6$. *$P < 0.01$ vs. untreated control (Ctl) by ANOVA and Student-Newman-Keuls test.

Fig. 3. Bleo does not alter steady-state levels of CatD mRNA. A: PCR products from 2 different primer sets (1 and 2, see MATERIALS AND METHODS) used to amplify CatD mRNA by RT-PCR; starting material was total RNA isolated from primary rat AECs exposed to Bleo (+) or vehicle (−) for the indicated times. B: real-time RT-PCR of CatD mRNA (primer set 2) at the indicated times after exposure to Bleo (see MATERIALS AND METHODS). B-MG, β-microglobulin; bars are means ± SE of 3 separate AEC cultures.

Fig. 4. Bleo increases the release of immunoreactive CatD protein from cultured AECs. Primary cultures of AECs were exposed to Bleo as in Fig. 2; detergent lysates were harvested from the cells (monolayer), and the cell culture medium was collected and concentrated. Equal amounts of lysate protein (10 µg/lane) or volume of culture medium (equivalent to 10$^5$ cells) were subjected to Western blotting with CatD-specific antibodies (see MATERIALS AND METHODS). Note increases in immunoreactive proteins of apparent molecular masses of ~52, 48, and 44 kDa (arrowheads) in medium from Bleo-treated AECs.

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sites for both CatD and ACE, which together generate ANG II.

To confirm this premise with the reagents currently commercially available for this study, the purified angiotensinogen fragment 1–14 (F1–14) was treated in vitro (without cells) with purified ACE or purified CatD enzymes (Fig. 7A). As expected, treatment of F1–14 with both ACE and CatD together resulted in significant production of the peptide ANG II (Fig. 7A), as measured by an ELISA that detects ANG II but not ANG I or angiotensinogen. In contrast, neither purified ACE alone nor purified CatD alone could convert F1–14 to the peptide ANG II.

In Fig. 8, incubation of primary rat AECs with F1–14 induced apoptosis detected by nuclear fragmentation. The apoptosis was completely blocked by the nonspecific or AT1-selective ANG receptor antagonists saralasin or L-158809, respectively. Moreover, the apoptosis was inhibited 96% by the CatD inhibitor pepA and was reduced 77% by neutralizing antibodies specific for CatD (both P < 0.001).
DISCUSSION

A role for the aspartyl protease CatD in apoptosis has been shown previously in HeLa cells exposed to interferon-γ, Fas ligand, or TNF-α (1) and in PA1 ovarian cancer cells (18). The activity of CatD is upregulated by the apoptosis inducer adriamycin in PA1 cells and in MLI leukemia cells and U1752 lung cancer cells (18). Although the aspartyl protease inhibitor pepA could block apoptosis in these cell types, the exact mechanism(s) by which CatD participates in the execution of apoptosis is unclear. In accord with the known ubiquitous expression of CatD as a lysosomal protease (10), it has been suggested that this and other lysosomal proteases might be involved in the production of a bioactive molecule required for apoptosis of PC12 cells in response to trophic withdrawal (5).

CatD also is known to be one of the enzymes capable of proteolytically processing the liver-derived and serum-borne protein angiotensinogen to the peptide ANG I, a function normally performed in the serum by the kidney-derived enzyme renin (2). On the other hand, evidence from several nonpulmonary cell types has established CatD as the primary enzyme that converts angiotensinogen to ANG I within local “intrinsic” ANG systems independently of renin (2, 17).

Recent studies from these laboratories have shown that Bleo-induced apoptosis of AEC requires the autocrine synthesis of angiotensinogen, ANG II, and its binding to ANG receptor AT1 (8). Those data were consistent with related studies showing that purified ANG II itself was a potent inducer of apoptosis in these cell types, the exact mechanism(s) by which CatD participates in the execution of apoptosis is unclear. In accord with the known ubiquitous expression of CatD as a lysosomal protease (10), it has been suggested that this and other lysosomal proteases might be involved in the production of a bioactive molecule required for apoptosis of PC12 cells in response to trophic withdrawal (5).

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The data herein strongly suggest that CatD functions in this capacity in AEC; Bleo-induced nuclear fragmentation and caspase-3 activity were significantly reduced by the aspartyl protease inhibitor pepA (Fig. 5) or by antisense oligonucleotides against CatD mRNA (Fig. 6). In earlier investigations, Bleo-induced apoptosis of AEC was completely blocked by specific ANG receptor antagonists or ANG-neutralizing antibodies (8); this finding lead to the theory that autocrine generation of ANG II is required for AEC apoptosis regardless of the initiating stimulus (11). In the light of those results, the finding that CatD antisense treatment did not completely block Bleo-induced nuclear fragmentation (48%, Fig. 6B) might indicate a potential role for additional protease(s) in angiotensinogen processing and subsequent AEC apoptosis. This interpretation is consistent with the finding that the protease inhibitor pepA, which blocks all aspartyl proteases, also was incapable of complete blockade of nuclear fragmentation (76%, Fig. 5) despite complete inhibition of CatD enzyme activity in AEC lysates (Fig. 2). On the other hand, the antisense treatment, which is at least theoretically specific, did not completely eliminate immunoreactive CatD detected by Western blotting (Fig. 6A). Thus it is difficult to know with certainty if the incomplete blockage of apoptosis is due to inefficient CatD knockdown or additional proteases activities.

Regardless, studies of angiotensinogen F1–14 (Figs. 7 and 8) are consistent with the theory that CatD is required for the conversion of angiotensinogen to ANG II by AEC and with earlier work. For example, the finding that incubation of primary rat AECs with F1–14 alone in serum-free culture medium (but without added enzymes) yielded significant production of ANG II (Fig. 7) is consistent with the earlier demonstration of constitutive, albeit low, expression of both ACE and an unidentified aspartyl protease by primary AECs (16). Moreover, the complete abrogation of AEC apoptosis in response to F1–14 by the nonselective and AT1-selective ANG receptor antagonists saralasin and losartan (Fig. 8) confirmed that the induction of apoptosis was dependent on both the generation of ANG II from F1–14 and the binding of ANG II to receptor AT1. Those results also are consistent with our earlier demonstrations that AT1 receptor mediates AEC apoptosis in response to Bleo (8), amiodarone (3, 13), or purified ANG II (9). Most important, the findings that AEC apoptosis in response to F1–14 was essentially abrogated by either pepA or by CatD antibodies (Fig. 8) strongly suggest that the conversion of angiotensinogen to ANG I, and subsequently ANG II to induce AEC apoptosis, is dependent on CatD activity.

The upregulation of CatD activity by Bleo in this study is consistent with the earlier findings that CatD is upregulated in AEC in fibrotic human lung (6) and is induced in the L132 lung cell line during apoptosis in vitro (7). In other cell types, apoptosis inducers upregulate both CatD protein and mRNA (18), which suggests control of activation at the level of RNA. In contrast, RT-PCR studies of AEC transcripts after Bleo treatment failed to detect changes in CatD mRNA (Fig. 3) despite significant increases in CatD activity (Fig. 2) and immunoreactive protein by Western blotting. It is possible that the relatively few sampling times chosen for real-time analyses of CatD mRNA may have missed a transient but short-lived increase in the mRNA that might be revealed by a more exhaustive time-course study. On the other hand, CatD is known to undergo activation by proteolytic mechanisms as well; in human U937 cells, CatD was shown to undergo processing of the inactive proproisoform (52 kDa) to the active pro-CatD (48 kDa) and an active 32-kDa isoform, in response to autocatalysis of the enzyme induced by the direct binding of the apoptosis mediator ceramide (4).

Consistent with those findings, Western blotting of rat AEC lysates did reveal Bleo-induced increases in several isoforms of apparent molecular mass 44–52 kDa. However, two of the isoforms shown to be increased in AEC media (52 and 48 kDa, see “medium” in Fig. 4) are larger than the primary isoform detected intracellularly in AEC (44 kDa, “monolayer” in Fig. 4). This finding argues against proteolytic processing alone as a mechanism of CatD activation in AEC. Thus the exact mechanism(s) by which Bleo upregulates CatD in AEC is unknown but will pose an interesting problem for future studies. pepA-inhibitable CatD activity also was upregulated by amiodarone (13) and TNF-α (14), both of which induce apoptosis in AECs (13, 14), but a determination of whether the requirement for CatD is universal to all proapoptotic stimuli for AEC will require further investigation.

In summary, Bleo upregulated CatD enzymatic activity and immunoreactive protein in primary cultures of rat AEC. Apoptosis of cultured AEC in response to Bleo was significantly inhibited by the aspartyl protease inhibitor pepA or by antisense oligonucleotides against CatD mRNA. The same inhibitors also prevented the enzymatic processing of a synthetic fragment of angiotensinogen (amino acids 1–14) and completely blocked AEC apoptosis in response to the same peptide.
These data are consistent with earlier studies showing that apoptosis of AEC in response to Bleo requires the autocrine synthesis and proteolytic processing of angiotensinogen to ANG II and suggest that the proteolytic processing requires CatD. The data herein also suggest that blockade of CatD and other aspartyl proteases might provide a potential strategy for preventing AEC apoptosis and lung injuries that involve this mode of cell death.

GRANTS

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