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Involvement of Cathepsin D in Apoptosis of Mammary Epithelial Cells

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ABSTRACT : During involution of the mammary gland after the lactation period, the gland undergoes an extensive epithelial cell death. In our previous study, overexpression of an extracellular proteinase inhibitor (Expi) gene accelerated apoptosis of mammary epithelial cells. Here we found that expression of the cathepsin D gene was induced in the Expi-overexpressed apoptotic cells. To understand the role of cathepsin D in apoptosis, we transfected cathepsin D gene into mammary epithelial HC11 cells and established the stable cell lines overexpressing the cathepsin D gene. We found that overexpression of the cathepsin D gene partially induced apoptosis of mammary epithelial cells. Expression patterns of the cathepsin D gene were examined in mouse mammary gland at various reproductive stages. Expression of the cathepsin D gene was increased during involution stages compared to lactation stages, and highest expression levels were shown at involution on day 4. We also examined expression of the cathepsin D gene in various mouse tissues. Mammary gland at involution on day 2 showed highest levels of cathepsin D mRNA of the mouse tissues that we examined. Liver tissues showed high levels of cathepsin D expression. These results demonstrate that cathepsin D may contribute to the apoptotic process of mammary epithelial cells. (**Key Words :** Cathepsin D, Apoptosis, Mammary Epithelial Cells)

INTRODUCTION

During involution of the mammary gland after the lactation period, the gland undergoes an extensive epithelial cell death (Strange et al., 1992; Walker et al., 1989). During the early phase of mammary gland involution, accumulation of milk is associated with an engorgement of the gland, with a marked change in the pattern of gene expression and with massive apoptosis of epithelial cells (Walker et al., 1989; Strange et al., 1992; Marti et al., 1999). Accumulation of factors in the milk, the change in shape of epithelial cells due to the engorgement, changes of hormone levels and loss of survival factor function are possible triggers of this initial phase of apoptosis (Topper and Freeman, 1980; Feng et al., 1995; Li et al., 1997; Marti et al., 1997, 1999). However, it remains unclear how these changes are translated into an apoptotic response in mammary epithelial cells. During the late phase of mammary gland involution, extracellular matrix degrading proteases are produced that may be responsible for the collapse of lobulo-alveolar structures and the subsequent tissue remodeling (Talhouk et al., 1992; Lund et al., 1996; Li et al., 1997).

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The induction of several genes including Fas antigen, 24P3, interleukin-10, lysozyme, and interleukin-1β converting enzyme has been reported during involution of the mammary gland and in apoptotic mammary epithelial HC11 cells (Strange et al., 1992; Boudreau et al., 1995; Lee et al., 2001; Bong et al., 2005). In a previous study, we observed induction of an extracellular proteinase inhibitor (Expi) gene in apoptotic mammary epithelial cells (Jung et al., 2004). The Expi gene was previously known as WDNM1 (Dear et al., 1988, 1989). Overexpression of the Expi gene accelerated apoptosis of mammary epithelial cells. Here we found that expression of cathepsin D was induced in the Expi-overexpressed apoptotic cells.

Cathepsin D is a lysosomal aspartic protease that is present in practically all animal cells, often in relatively high concentrations (Yamamoto, 1995). This enzyme degrades proteins at low pH and is believed to play important roles in protein catabolism, antigen-processing, protein targeting, and the progression of breast cancer (Rochefort et al., 1996). The participation of lysosomal proteases in apoptosis has long been neglected, because lysosomes appear to remain intact during apoptosis, and release of lysosomal content has therefore been related to necrotic cell death (Zhivotovsky et al., 1997). Some studies have shown that the cathepsin D gene is activated during

apoptosis induced by TNF-a, Fas, interferon-gamma, and Adriamycin (Diess et al., 1996; Wu et al., 1998).

There are no data available for cathepsin D expression and its function in mammary gland. To examine the role of cathepsin D in apoptosis, cathepsin D cDNA was transfected into mammary epithelial HC11 cells, and percent apoptosis was determined in the cathepsin D-transfected cells. Expression patterns of cathepsin D gene were examined in mouse mammary gland at various reproductive stages.

MATERIALS AND METHODS

Mammary epithelial HC11 cell culture and transfection of cathepsin D cDNA

The HC11 cell is a clonal mammary epithelial cell that is derived from spontaneously immortalized COMMA-D epithelial cells, isolated from the mammary gland of midpregnant BALB/c mice (Ball et al., 1988). HC11 cells were cultured in RPMI1640 growth medium (Gibco BRL, USA) containing 10% fetal bovine serum (Gibco BRL), 5 μg/ml insulin, 10 μg/ml epidermal growth factor (EGF), and 50 μg/ml gentamycin (Sigma, USA) in a 5% CO_2 at 37°C. For apoptotic cells, confluent cells were cultured in medium containing 2% FBS and insulin for 2 days and incubated in serum-free medium without insulin and EGF for 2 days (Han et al., 2005). Previously, we have developed cell lines overexpressing Expi gene (Jung et al., 2004), and we used the Expi-transfected cells in the current study.

For construction of cathepsin D expression vector, the cathepsin D cDNA was amplified by RT (reverse transcriptase)-PCR using total RNA templates isolated from 4 d involuted mammary gland. The following primers were used for PCR:

Cathepsin D forward primer: 5' accatgaagactcccggcgtc 3' ; 21mer
Cathepsin D reverse primer: 5' gagtacgacagcattggcaaa 3' ; 21mer

The 5' primer started from 3 nucleotide sequences ahead of the start codon, and reverse primer was designed to prime amino acid leucine (ctc) just before the stop codon, thus translating through vector sequences V5-His. PCR reaction was performed using Taq polymerase for 30 cycles at 55°C annealing temp. Cathepsin D expression vector was constructed using pcDNA3.1/V5-His-TOPO vector. PCR products and pcDNA3.1/V5-His-TOPO vector were mixed, and TOPO cloning reaction was performed for 5 min at room temp. Reaction mixtures were transformed into TOP10 *E. coli* cells. Constructs were confirmed by BstX I digestion and by nucleotide sequencing of entire coding region of the cDNA and junction region of the vector. For transfection, plasmid DNA was isolated by QIAGEN endo free kit (QIAGEN).

The HC11 cells (1.0×10^6) were seeded in each 6-well plate in the growth medium without antibiotics, and incubated until 90% confluency for one day. The recombinant plasmid (4.0 μg) was transfected using lipofectamine 2000 according to the manufacturer's instruction (Gibco BRL). For stable cell lines, transfected cells were selected by adding the medium containing 200 μg/ml Geneticin (G418; Gibco BRL). The medium was changed every 48 h. After 14 days of G418 selection, colonies were obtained by trypsinization of a colony within a cloning cylinder (Sigma). The cells were transferred onto 24 well dishes and cultured in the medium containing 10% serum until confluency. The cells were transferred onto 6 well dishes and propagated.

Genomic DNA was isolated from the transfected cells. The cells were cultured until confluency, washed twice by 1×TBS buffer and scraped. The cells were lysed using DNA extraction buffer and proteinase K. After reaction, tris phenol (25): chloroform (24): isoamylalcohol (1) solution was added. The pellet of genomic DNA was precipitated by 99% ethanol, washed with 70% ethanol and dried. The DNA pellet was dissolved in TE buffer. To confirm pcathepsin D integration into the genomic DNA of cells, PCR amplification was performed with the isolated genomic DNA and CMV 5' primer and cathepsin D reverse primer for 35 cycles (preheating at 94°C for 5 min; cycling at 94°C for 30 sec, at 55°C for 1 min 30 sec and at 72°C for 1 min 30 sec; final elongation at 72°C for 10 min). The PCR products were checked by 1.0% agarose gel electrophoresis.

The 4,6-diamidine-2'-phenylindole dihydrochloride (DAPI; Sigma) staining was performed as described previously (Lowe et al., 1993; Jung et al., 2004). Briefly, media was removed, cells were washed in 1X PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, then washed with PBS. The cells were treated with 0.5% triton X-100/PBS for 5 min at room temperature for permeabilization of the cells. The cells were stained with 1 μg/ml DAPI/PBS for 30 min at room temperature. Cells were examined by fluorescence inverted microscope, and apoptotic cells were identified by condensation and fragmentation of nuclei. A minimum of 400 cells was counted for each well, and percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells.

Tissue sampling and northern analysis

The ICR mouse mammary membrane (Seegene, Korea), which is pre-made for immediate use, was analyzed for northern analysis. Various tissues from ICR mouse were collected, and total RNA was extracted by the acid guanidinium thiocyanate phenol/chloroform method

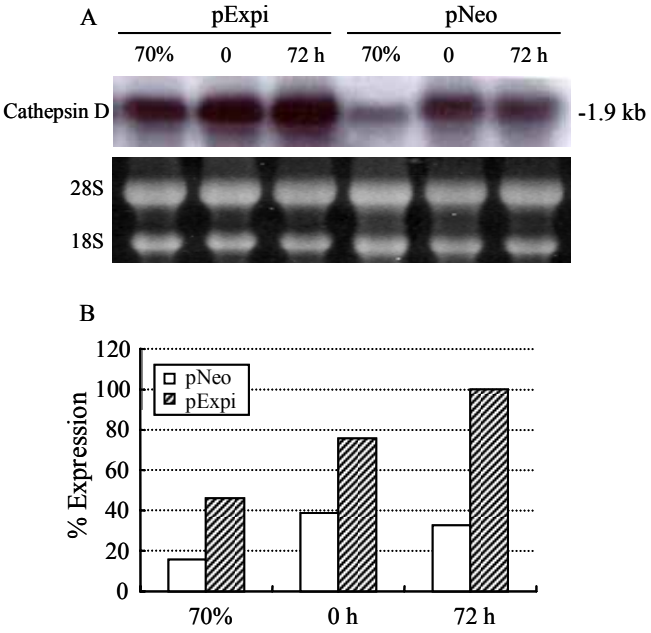

Figure 1. Expression of cathepsin D gene in the Expi-transfected HC11 cells. A: The pExpi and pNeo plasmids were transfected in HC11 cells, and stable cell lines overexpressing Expi gene were established previously (Jung et al., 2004). The cells were grown to confluency in growth medium containing EGF, insulin and 10% FBS, kept for 2 days in the medium containing 2% FBS but neither insulin nor EGF. The cells were incubated for 0 and 72 h in serum-free medium, and total RNA was prepared from cells. The total RNA was also prepared from the 70% confluent cells cultured in growth media (70%). mRNA levels were determined by northern analysis using ^{32}P-labeled cDNA probe. The 28S and 18S rRNAs were shown as loading control. B: mRNA levels were quantitated by phosphoimage analyzer. Values of percent expression (mRNA levels of cathepsin D/28S) were normalized to 100 for the highest expression levels.

(Chomczynski and Sacchi, 1987). Twenty micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, and blotted onto a membrane.

RT-PCR products of cathepsin D were purified using AccuPrep^{TM} PCR purification kit (Bioneer, Korea). Purified PCR products were cloned into TA cloning vector, pCR2.1 (Invitrogen, USA), and correct nucleotide information was confirmed by nucleotide sequencing. The plasmid was digested with EcoR I, and the insert was obtained after low melting agarose gel electrophoresis. The insert of the cDNA clone was labeled using a Prime-It Random Primer Labeling Kit (Stratagene). The membrane was hybridized with the ^{32}P-labeled insert of the cDNA clone. The equal amount of RNA loading was confirmed by the intensities of 28S and 18S band, and the efficiency of transfer was monitored by ethidium bromide staining.

The membrane was prehybridized with the prehybridization solution (10%% dextran sulfate, 0.5% SDS, 6×SSC, 1 mM EDTA, 100 μg/ml salmon sperm DNA)

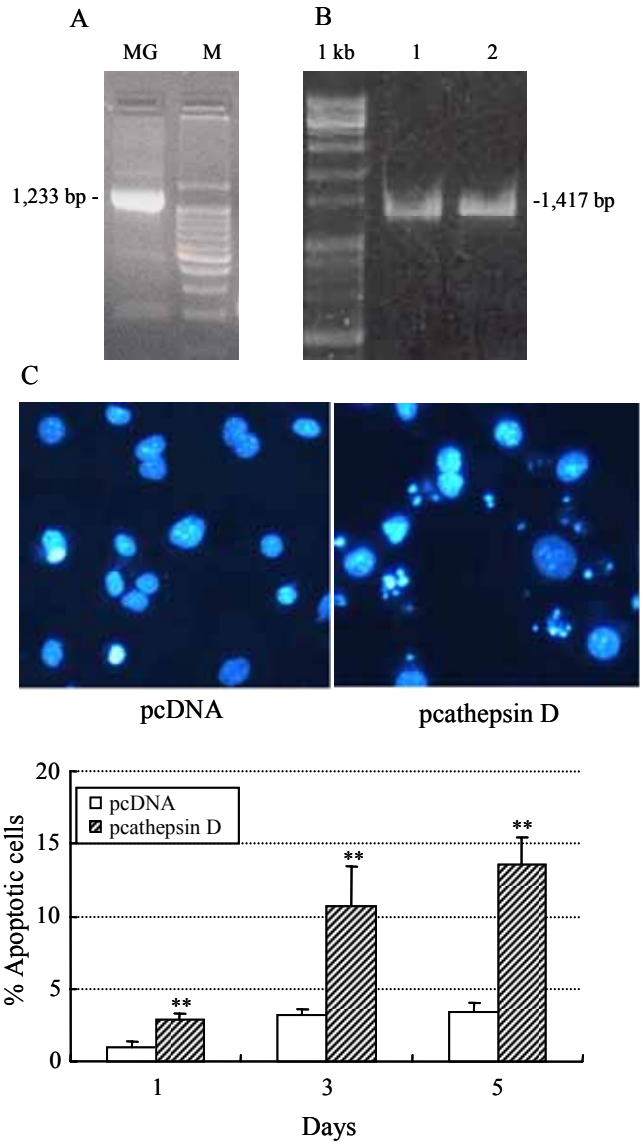

Figure 2. Transfection of cathepsin D cDNA into the HC11 cells and induction of apoptosis in the cathepsin D-transfected cells. A: The cathepsin D cDNA was amplified by RT-PCR using mammary gland (MG) total RNA as templates, and cathepsin D cDNA expression vector was constructed as described in materials and methods. M, 100 bp ladder. B: The pcathepsin D and control pcDNA plasmids were transfected in HC11 cells, and stable cell lines overexpressing cathepsin D gene were established, and integration of cathepsin expression vector for clones 1 and 2 was confirmed by PCR using genomic DNA as templates. The 1,417 bp band containing the 1,233 bp cathepsin D cDNA plus 184 bp vector sequences was generated by using CMV 5' vector primer and cathepsin D reverse primer. 1 kb, 1 kb ladder. C: Apoptotic cells were examined by DAPI staining at 1, 3, and 5 days in serum-free medium by a fluorescence-inverted microscope. Representative images are shown. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells. Data are the means+standard deviation of three independent experiments. ** indicates significant differences ($p<0.01$).

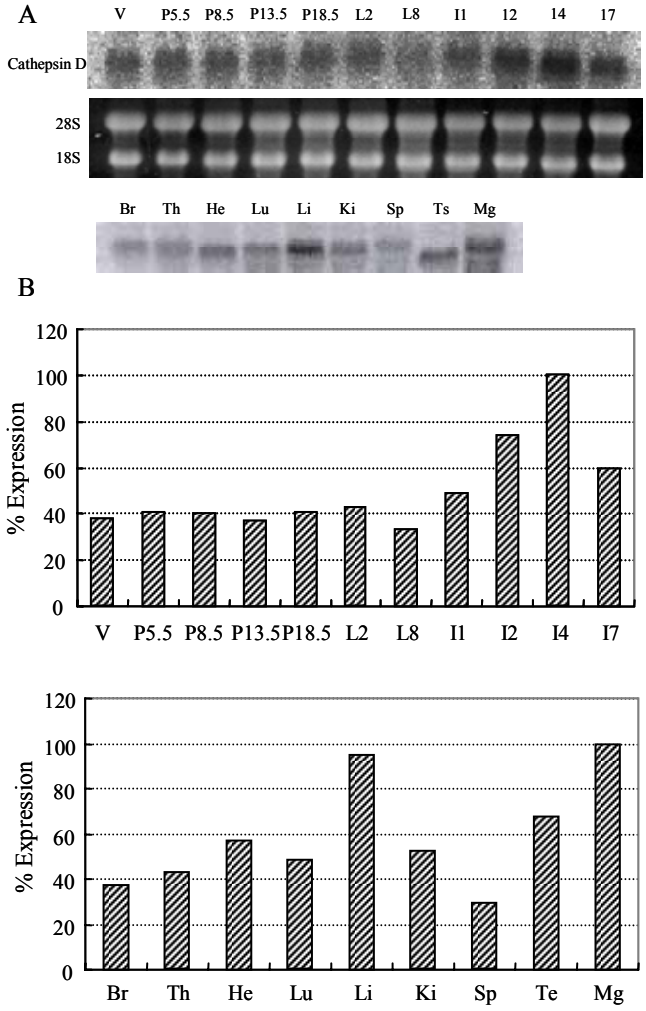

Figure 3. Northern analysis of cathepsin D gene in mouse tissues. A: Top: the RNA blot prepared at virgin (V), pregnant 5.5, 8.5, 13.5, and 18.5 days (P5.5, P8.5, P13.5, P18.5), lactation 2 and 8 days (L2, L8), and involution 1, 2, 4, and 7 days (I1, I2, I4, I7) in mouse mammary gland was hybridized with ^{32}P-labeled cDNA probe. The 28S and 18S rRNAs were shown as loading control. Bottom: the RNA blot prepared from brain (Br), thymus (Th), heart (He), lung (Lu), liver (Li), kidney (Ki), spleen (Sp), testis (Te), and mammary gland at involution days 2 (Mg) of mouse was analyzed by northern method. B: mRNA levels were quantitated by phosphoimage analyzer. Values of percent expression (mRNA levels of cathepsin D/28S) were normalized to 100 for the highest expression levels.

at 65°C for 1 h, the cDNA probe, preheated at 95°C, was added, and hybridization was performed at 65°C for 20 h. The membranes were washed twice in 2×SSC/0.1% SDS at room temperature for 10 min, once in 2×SSC/0.1% SDS at 42°C for 30 min, once in 0.1×SSC/0.1% SDS at 42°C for 30 min, once in 0.1×SSC/0.1% SDS at 55°C for 30 min, and once in 0.1×SSC/0.1% SDS at 68°C for 30 min. The membranes were exposed to phosphoimage cassette at room temperature for 24-48 h.

RESULTS AND DISCUSSION

Expression of cathepsin D gene in Expi-transfected mammary epithelial cells

Previously, we developed the stable cell lines overexpressing Expi gene, and we found that the overexpression of the Expi gene accelerated the apoptosis of mammary epithelial cells under serum starvation (Jung et al., 2004). In the current study, expression levels of cathepsin D gene were examined by northern analysis in the Expi-transfected cells at 70% confluent stage and 0 h and 72 h incubation in serum-free media. Expi-transfection showed an upregulation (2-3 fold) of cathepsin D gene expression at 70% confluent stage and 0 h and 72 h in serum-free media (Figure 1).

Induction of apoptosis in cathepsin D-transfected cells

To understand the function of cathepsin D in the apoptosis of mammary epithelial cells, cathepsin D expression vector was constructed using pcDNA3.1/V5-His-TOPO vector and cathepsin D cDNA products (1,233 bp) obtained by RT-PCR amplification of mammary gland total RNA (Figure 2A). The recombinant DNA was transfected into HC11 cells using the lipofectamine method. We developed the stable cell lines overexpressing the cathepsin D gene. After two weeks of G418 selection, we isolated four colonies of cathepsin D- and two colonies of cDNA3.1E-transfected cells. Integration of cathepsin D gene was confirmed by PCR using genomic DNA and CMV5' and cathepsin D3' primers. Expected 1.4kb fragments were detected in the cathepsin D-transfected cells (Figure 2B). Apoptotic cells were determined in the cathepsin D-transfected cells. The cells were grown to confluency in growth medium containing EGF, insulin and 10% FBS, and kept for 2 days in the medium containing 2% FBS but neither insulin nor EGF. The cells were incubated for 1, 3, and 5 days in serum-free medium. The percent apoptotic cells was less than 5% in control cDNA3.1E-transfected cells at 1 d, 3 d, and 5 d in serum-free medium. Cathepsin D transfection increased the percent apoptotic cells by 7% and 10% at 3 d and 5 d in serum-free medium, respectively (Figure 2C). There was no induction of apoptosis in cathepsin D-overexpressed cells in the presence of serum. Serum may have survival factor(s) that prevents induction of apoptosis. This result demonstrates that overexpression of cathepsin D partially induces apoptosis of mammary epithelial cells.

Expression of cathepsin D gene in mouse tissues

Expression pattern of cathepsin D gene was examined in mouse mammary gland in various physiological conditions including virgin, pregnancy (5.5, 8.8, 13.5 and 18.5 days), lactation (2 and 8 days), and involution stages (1, 2, 4, and 7 days). Expression levels of cathepsin D gene were

relatively constant at virgin through late pregnancy with a moderate level, the expression was decreased at lactation stage, then the expression was highly induced from day 2 of involution and peaked at day 4 of involution, and thereafter expression levels were decreased (Figure 3). We examined expression of cathepsin D gene at various mouse tissues. Mammary gland at day 2 of involution showed the highest levels of cathepsin D mRNA of the mouse tissues that we examined (Figure 3). Liver tissues also showed high levels of expression. Other tissues including brain, thymus, heart, lung, kidney and testis showed a moderate level of cathepsin D expression.

It has been suggested that lysosomes and lysosomal enzymes are necessary for the onset of apoptosis induced by oxidative stress. Furthermore, lysosomal destabilization was observed, detected as a release of cathepsin D, suggesting that this is an essential early event in apoptosis and that it occurs "upstream of" the release of mitochondrial cytochrome c (Roberg, 2001). A clear role for these proteases and the acidic endolysosomal compartment in apoptotic signaling is not yet defined. A recent study suggests that cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation (Heinrich et al., 2004). Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis (Bidere et al., 2003). We found that overexpression of the cathepsin D gene partially induced apoptosis of mammary epithelial cells. High induction of the cathepsin D gene was observed in involution stages of mouse mammary gland at which an extensive mammary epithelial cell death occurs by apoptosis. Detailed study of the role of cathepsin D in apoptosis of mammary epithelial cells is needed.

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