

# Cathepsin E Prevents Tumor Growth and Metastasis by Catalyzing the Proteolytic Release of Soluble TRAIL from Tumor Cell Surface

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## Abstract

The aspartic proteinase cathepsin E is expressed predominantly in cells of the immune system and highly secreted by activated phagocytes, and deficiency of cathepsin E in mice results in a phenotype affecting immune responses. However, because physiologic substrates for cathepsin E have not yet been identified, the relevance of these observations to the physiologic functions of this protein remains speculative. Here, we show that cathepsin E specifically induces growth arrest and apoptosis in human prostate carcinoma tumor cell lines without affecting normal cells by catalyzing the proteolytic release of soluble tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) from the cell surface. The antitumor activity of cathepsin E was corroborated by *in vivo* studies with mice bearing human and mouse tumor transplants. Administration of purified cathepsin E into human tumor xenografts in nude mice dose-dependently induced apoptosis in the tumor cells to inhibit tumor growth. The growth, viability, and metastasis of mouse B16 melanoma cells were also more profound in cathepsin E–deficient mice compared with those in the syngeneic wild-type and transgenic mice overexpressing cathepsin E. Taken together, the number of apoptotic tumor cells, as well as tumor-infiltrating activated macrophages, was apparently reduced in cathepsin E–deficient mice compared with those in the other two groups, implying the positive correlation of endogenous cathepsin E levels with the extent of tumor suppression *in vivo*. These results thus indicate that cathepsin E plays a substantial role in host defense against tumor cells through TRAIL-dependent apoptosis and/or tumor-associated macrophage-mediated cytotoxicity. [Cancer Res 2007;67(22):10869–78]

## Introduction

The proteolytic activity of various types of proteases, including lysosomal cathepsins (B, L, and D) and matrix metalloproteinases (MMP-1, MMP-9), have long been associated with many types and stages of cancer (reviewed in ref. 1, 2) and thus thought to be attractive cancer targets. However, several strategies designed to

broadly block such proteases have been unsuccessful due in part to their functional diversity *in vivo*. Intriguingly, some of the MMP family members, such as MMP-3, MMP-8, and MMP-12, were found to have antitumorigenic effects through the suppression of tumor angiogenesis and degradation of chemokines that mediate organ-specific metastasis (2). Cathepsin E is an endolysosomal aspartic proteinase that is expressed predominantly in cells of the immune system (3–5) and is highly secreted by activated phagocytes (3). Unlike the analogous aspartic proteinase cathepsin D, cathepsin E possesses notable properties (3), including limited distribution, cell-specific localization, and cell-specific processing. It has also been shown that cathepsin E plays a role in the processing of exogenous antigens for their presentation by MHC class II molecules (4, 6, 7), and that cathepsin E knock-out (*CatE*<sup>−/−</sup>) mice exhibit impaired immune responses (8, 9). Cathepsin E deficiency was also shown to induce a novel form of lysosomal storage disorder exhibiting the accumulation of major lysosomal membrane sialoglycoproteins such as LAMP-1 and LAMP-2 and the elevation of lysosomal pH in macrophages, thereby resulting in the impairment of their structural and functional integrity (10). Thus, cathepsin E is more likely to contribute to the maintenance of homeostasis by participating in host defense mechanisms. Recent gene or protein expression profiling of certain cancers also suggests the clinical utility of cathepsin E as a potential cancer biomarker (11–14). However, because physiologic substrates of cathepsin E have not yet been identified, the relevance of these observations to the physiologic functions of this protein remains speculative.

Apoptosis contributes to the elimination of transformed or virally infected cells during immune responses and is therefore a major determinant of the growth or regression of tumors. Accumulating evidence indicates that apoptosis in tumor cells is regulated by ligand-type cytokine molecules of the tumor necrosis factor (TNF) family, such as TNF- $\alpha$ , FasL, and TNF-related apoptosis-inducing ligand (TRAIL; ref. 15). Unlike other TNF family members, TRAIL can induce apoptosis in a wide variety of tumor cells without affecting normal cells (16, 17). Previous studies showed that not only the cell-associated form of TRAIL, but also the soluble form led to a higher induction of apoptosis in tumor cells (16, 18, 19). Because of the utility of TRAIL as a potential therapy to treat human cancer, it seems to be of importance to identify and validate protease(s) responsible for the generation of soluble TRAIL from the cell surface of various cell types. To date, however, only limited data are available about the identity of the proteolytic enzyme(s) involved in TRAIL cleavage (20). Hence, to determine whether cathepsin E has a role in multistage processes important for tumorigenesis and, if so, what the mechanism for manifestation of its action is, we manipulate this protein in both

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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*in vitro* and *in vivo* experiments using human tumor cell lines, nude mice bearing human tumor cells, and *CatE*<sup>-/-</sup> or cathepsin E-overexpressing transgenic mice (*CatE*<sup>Tg</sup>) bearing syngeneic mouse melanoma cells.

## Materials and Methods

**Cells.** Four human prostate carcinoma cell lines (ALVA-41, ALVA-101, PPC-1, and DU145) were kindly provided by J.Y. Bahk (Gyeongsang National University, Korea). The human prostate carcinoma cell line PC-3 and normal human prostate epithelial (PrE) cells were obtained from Dainippon. Mouse B16 melanoma cells were obtained from RIKEN BioResource Center.

**Measurement of cell viability.** Cell viability was determined with the use of a Cell Counting Kit-8 (Dojindo Molecular Technologies). Cell viability was also examined by phase-contrast microscopy for morphologic signs of apoptosis. Cells in the early and late stages of apoptosis were detected by staining with annexin V and by the terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay, respectively.

**Preparation of natural and recombinant cathepsins.** Natural cathepsins B, L, D, and E were purified from rat sources as previously described (21). Recombinant wild type and its mutant protein (D98A/D283A) in which two aspartate residues in the active site are replaced with alanine were expressed in HEK293T cells and purified according to the method previously described (22). The active enzyme concentrations of aspartic cathepsins (D and E) and cysteine cathepsins (B and L) were determined by titration against the competitive inhibitor pepstatin A and E-64, respectively.

**DEAE-Sephacel chromatography and immunodepletion.** ALVA-41 cells ( $9 \times 10^9$ ) that had been incubated for 20 h at 37°C with cathepsin E (1.2 μmol/L) in serum-free OptiMEM were separated by centrifugation. The supernatant was concentrated and dialyzed against 10 mmol/L sodium phosphate buffer (pH, 7.0) containing 0.05% Brij 35 and then applied to a column of DEAE-Sephacel equilibrated with the same solution. The column was subjected to stepwise elution with NaCl. The cytotoxic and cathepsin E activities of fractions were determined with freshly prepared ALVA-41 cells and the cathepsin E-specific substrate MOCAc-Gly-Ser-Pro-Ala-Phe-Leu-Ala-Lys(Dnp)-D-Arg-NH<sub>2</sub> (21), respectively. For immunodepletion of TRAIL, the 0.1-mol/L NaCl fraction was incubated overnight at 4°C first with mouse monoclonal antibodies to TRAIL (Sigma-Aldrich Inc.) or with control mouse immunoglobulin G (IgG; 1 μg/mL for each) and then with protein G-Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences). The resin was removed by centrifugation, and the resulting supernatant was evaluated for its effect on the viability of ALVA-41 cells.

**ELISAs for TRAIL, FasL, and TNF-α.** The amounts of TRAIL, FasL, or TNF-α in culture supernatants of ALVA-41 cells ( $1 \times 10^6$  per 6-cm dish) that had been incubated for 20 h at 37°C with purified cathepsin E in serum-free OptiMEM were measured with the use of ELISA kits (Biosource International).

**Immunohistochemistry.** Formalin-fixed and paraffin-embedded xenograft sections were examined for F4/80 and MHC class II using the CHEMICON IHC Select Immunoperoxidase Secondary Detection System according to the manufacturer's instruction. Sections were counterstained with hematoxylin and examined under an optical microscope.

**Animals.** All animals were maintained according to the guidelines of the Japanese Pharmacological Society in a specific pathogen-free facility at the Kyushu University Station for Collaborative Research. All animal experiments were approved by the Animal Research Committee of the Graduate School of Dental Science, Kyushu University. Wild-type and *CatE*<sup>-/-</sup> mice on the C57BL/6 genetic background were used as described previously (8). Transgenic mice overexpressing cathepsin E (*CatE*<sup>Tg</sup>) were generated according to the method as described in Supplementary Methods (Supplementary Fig. S1). Cathepsin E mRNA levels in almost all tissues of *CatE*<sup>Tg</sup> mice were significantly higher than those in wild-type littermates (Supplementary Fig. S2). *CatE*<sup>Tg</sup> mice were fertile and exhibited normal bleeding behavior and no obvious phenotypes when raised under specific pathogen-free conditions.

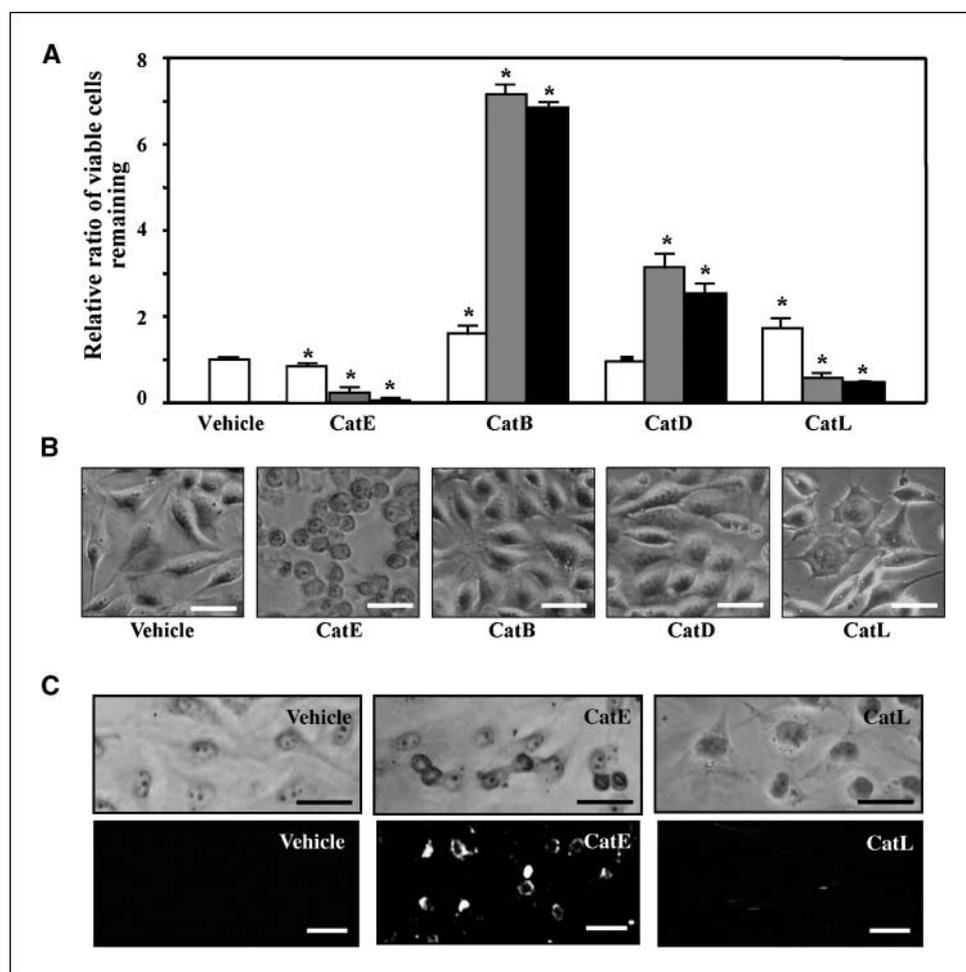
**Determination of tumor growth and metastasis in mice bearing human and mouse xenografts.** For studies of primary tumor growth, ALVA-41 cells ( $5 \times 10^6$ ) in 0.1 mL of PBS were injected s.c. into the right flank of the male nude mice. Similarly, to determine the association of endogenous cathepsin E expression levels with the suppression of tumor growth, metastasis, and mortality of syngenic tumor-bearing animals, B16 melanoma cells ( $1 \times 10^6$ ) in 0.1 mL of PBS were injected s.c. into syngenic wild-type, *CatE*<sup>-/-</sup>, and *CatE*<sup>Tg</sup> mice. Tumor size was measured with calipers, and tumor volume was calculated as  $ab^2/2$ , where  $a$  and  $b$  are the largest and smallest central cross-sectional dimensions, respectively. When the size of ALVA-41 xenografts had reached about 100 mm<sup>3</sup> (~12 days after injection), the animals were randomly divided into two groups of five, and purified cathepsin E (2.5 nmol/L/kg/day) or vehicle (saline) was given into the central region of the tumor for 16 days. For measurement of tumor weight and immunohistochemical studies, cathepsin E (5 nmol/L in polyethylene glycol/kg/day) was given into the central region of the tumor for 10 days after tumor size had reached about 400 mm<sup>3</sup>. For studies of metastasis, B16 cells ( $2 \times 10^5$  in 50 μL of PBS) were injected into the tail vein of each genotype. The animals were anesthetized and killed with a lethal dose of diethyl ether 22 days later, the lungs were removed, and the number of metastatic black nodules on the lung surface was counted.

## Results

**Effects of various cathepsins on cell viability and apoptosis in human prostate carcinoma ALVA-41 cells.** We first optimized the concentration of various cathepsins to determine their effects on tumor cell viability. After several trials, we found that cathepsins B, L, D, and E had optimal effects on the cell viability at approximately equimolar levels. Based on these results, we examined the possible cytotoxic effects of these cathepsins on the human prostate carcinoma cell line ALVA-41 by incubating the cells with each protease for 20 h at neutral pH *in vitro*. Cathepsins B and D each increased the number of viable cells in a concentration-dependent manner (Fig. 1A). Cathepsin L exhibited a biphasic effect on these cells, increasing the viable cell number at low concentration (0.25 μmol/L) and reducing it at high concentrations (1.2 and 2.4 μmol/L). Cathepsin E induced a concentration-dependent decrease in the number of viable ALVA-41 cells. At the microscopic level, cells treated with cathepsins B or D exhibited a morphology characteristic of growth phase, whereas those exposed to cathepsin E manifested morphologic hallmarks of apoptosis (Fig. 1B). Cells treated with cathepsin L (2.4 μmol/L) showed morphologic changes typical of necrotic damage, such as swelling and disintegration of cellular and nuclear structure. To confirm the difference in the pattern of cell damage induced by cathepsins E and L, we analyzed cells treated with these proteases by staining with annexin V and by the TUNEL assay. Cells treated with cathepsin E were positive for staining both with annexin V and with the TUNEL reagents, whereas those treated with cathepsin L were negative for both types of staining (Fig. 1C), thus confirming cathepsin E-induced apoptosis and cathepsin L-induced necrosis.

The cytotoxicity of cathepsin E was blocked by the addition of the aspartic protease inhibitor pepstatin A to the culture medium of ALVA-41 cells (Fig. 2A). Consistent with this, a cathepsin E mutant (D98A/D283A) had no effect on human embryonic kidney (HEK)293T cell viability, whereas the corresponding wild-type recombinant protein, likewise natural cathepsin E, exhibited concentration-dependent cytotoxicity on both HEK293T cells (Fig. 2B) and ALVA-41 cells (data not shown). These results thus indicate that the induction of apoptosis by cathepsin E is completely dependent on its catalytic activity. To determine whether apoptosis induction by cathepsin E was due to a direct

**Figure 1.** Effects of various cathepsins on the viability of human prostate carcinoma ALVA-41 cells. **A**, the cells ( $1 \times 10^4$ ) were seeded in 96-well plates in a volume of 100  $\mu$ L and cultured for 24 h. Then the cells were incubated for 20 h at 37°C and pH 7.4 with vehicle or with cathepsins B (0.4  $\mu$ mol/L, open column; 2  $\mu$ mol/L, gray column; 4  $\mu$ mol/L, black column), D (0.2  $\mu$ mol/L, open column; 1  $\mu$ mol/L, gray column; 2  $\mu$ mol/L, black column), E (0.1  $\mu$ mol/L, open column; 0.5  $\mu$ mol/L, gray column; 1  $\mu$ mol/L, black column), or L (0.25  $\mu$ mol/L, open column; 1.2  $\mu$ mol/L, gray column; 2.4  $\mu$ mol/L, black column) in serum-free OptiMEM, after which 10  $\mu$ L of the Cell Counting Kit-8 were added to each well, and the cells were incubated for an additional 1 h. The number of viable cells was determined with a colorimetric assay. Data are expressed relative to the value of vehicle-treated ones and are the means  $\pm$  SD of values from four independent experiments. \*,  $P < 0.001$ , versus the corresponding value for cells treated with vehicle. **B**, microscopic observations of the cells treated with cathepsins E (1  $\mu$ mol/L), B (4  $\mu$ mol/L), D (2  $\mu$ mol/L), L (2.4  $\mu$ mol/L) or vehicle. Bars, 50  $\mu$ m. **C**, after treatment with cathepsin E (0.5  $\mu$ mol/L), cathepsin L (2.4  $\mu$ mol/L), or vehicle at pH 7.4 for 20 h at 37°C or for 4 h at 37°C, the cells were isolated by centrifugation, washed twice with PBS, and then subjected to the TUNEL assay with an Apop Tag Peroxidase Apoptosis Detection Kit (Intergen; top) or to staining with FITC-labeled annexin V (Sigma-Aldrich Japan; bottom) for the detection of 22apoptotic cells. Bars, 50  $\mu$ m.



or indirect action on tumor cells, the culture supernatant of cathepsin E-treated ALVA-41 cells was added to freshly prepared cell cultures in the absence or presence of pepstatin A. This supernatant induced a decrease in cell viability in the presence of pepstatin A in a similar manner as that was apparent in the absence of this agent, and the cell viability loss was dependent on the concentration of cathepsin E in the initial culture (Fig. 2C), suggesting that cathepsin E-induced apoptosis is mediated by a certain molecule(s) released from the tumor cell surface by the catalytic action of cathepsin E. To test this further, we subjected the culture supernatant of cathepsin E-treated cells to DEAE-Sephacel chromatography. More than 80% of cathepsin E activity was detected in the column fraction eluted with 0.3 mol/L NaCl, as described previously (23), whereas most of the apoptosis-inducing activity was found in the fraction eluted with 0.1 mol/L NaCl, in which cathepsin E activity was barely detectable (Fig. 2D), indicating the separation of the apoptosis-inducing molecule from cathepsin E.

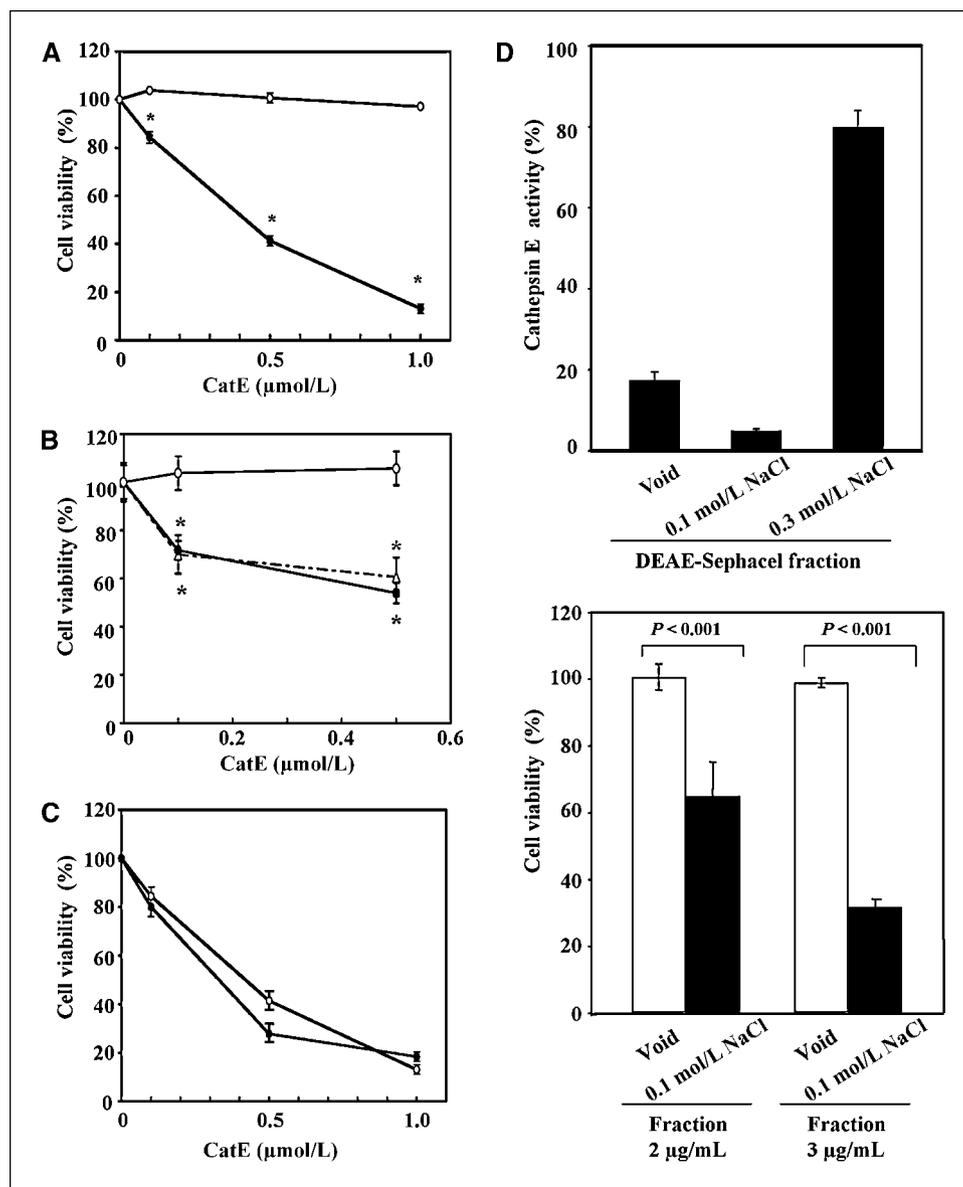
**Identification of TRAIL as a mediator responsible for cathepsin E-mediated tumor cell apoptosis.** To assess the possible involvement of TNF family members in cathepsin E-mediated apoptosis in ALVA-41 cells, the ELISA assay was done using specific antibodies against each ligand. As shown in Fig. 3A, TRAIL was increased in the culture supernatant of cathepsin E-treated cells, whereas little or no TNF- $\alpha$  and FasL were found in the culture supernatant. Given that the trimerization of the

extracellular COOH-terminal region of TRAIL, like other TNF family members, is necessary for the induction of the apoptotic signaling cascade through binding to receptors on the target cell surface (24–27), the soluble form of TRAIL released from ALVA-41 cells by cathepsin E was analyzed further by SDS-PAGE under reducing conditions and immunoblot analysis with antibodies to TRAIL. Two intense immunoreactive bands with apparent molecular masses of 63 and 65 kDa and a minor band at 48 kDa were detected in the culture supernatant of cathepsin E-treated cells but not in that of vehicle-treated cells (Fig. 3B). Given that the molecular mass of the extracellular portion of TRAIL calculated on the basis of its amino acid sequence is 21 kDa, and that the soluble, trimeric TRAIL is stabilized by a zinc ion interacting with a unique cysteine residue (Cys<sup>230</sup>) in the molecule (28), the 63- and 65-kDa proteins and the 48-kDa protein detected in the medium of cathepsin E-treated cells likely correspond to trimeric and dimeric forms of soluble TRAIL, respectively. Consistent with ELISA data, little generation of soluble trimeric forms of TNF- $\alpha$  or FasL was observed in the cathepsin E-treated cells. No trimeric form of soluble TRAIL was also generated by ALVA-41 cells treated with cathepsins D, B, or L (data not shown). To determine whether the soluble form of TRAIL generated by cathepsin E accounts for all of the apoptosis-inducing activity in the culture supernatant of cathepsin E-treated cells, we examined the effect of depletion of TRAIL on the cytotoxicity of the 0.1-mol/L NaCl fraction from DEAE-Sephacel chromatography. Immunodepletion of TRAIL with

its antibodies from this fraction blocked its apoptosis-inducing activity (Fig. 3C), indicating that the cathepsin E-mediated apoptosis is due mostly to TRAIL released from the tumor cell surface.

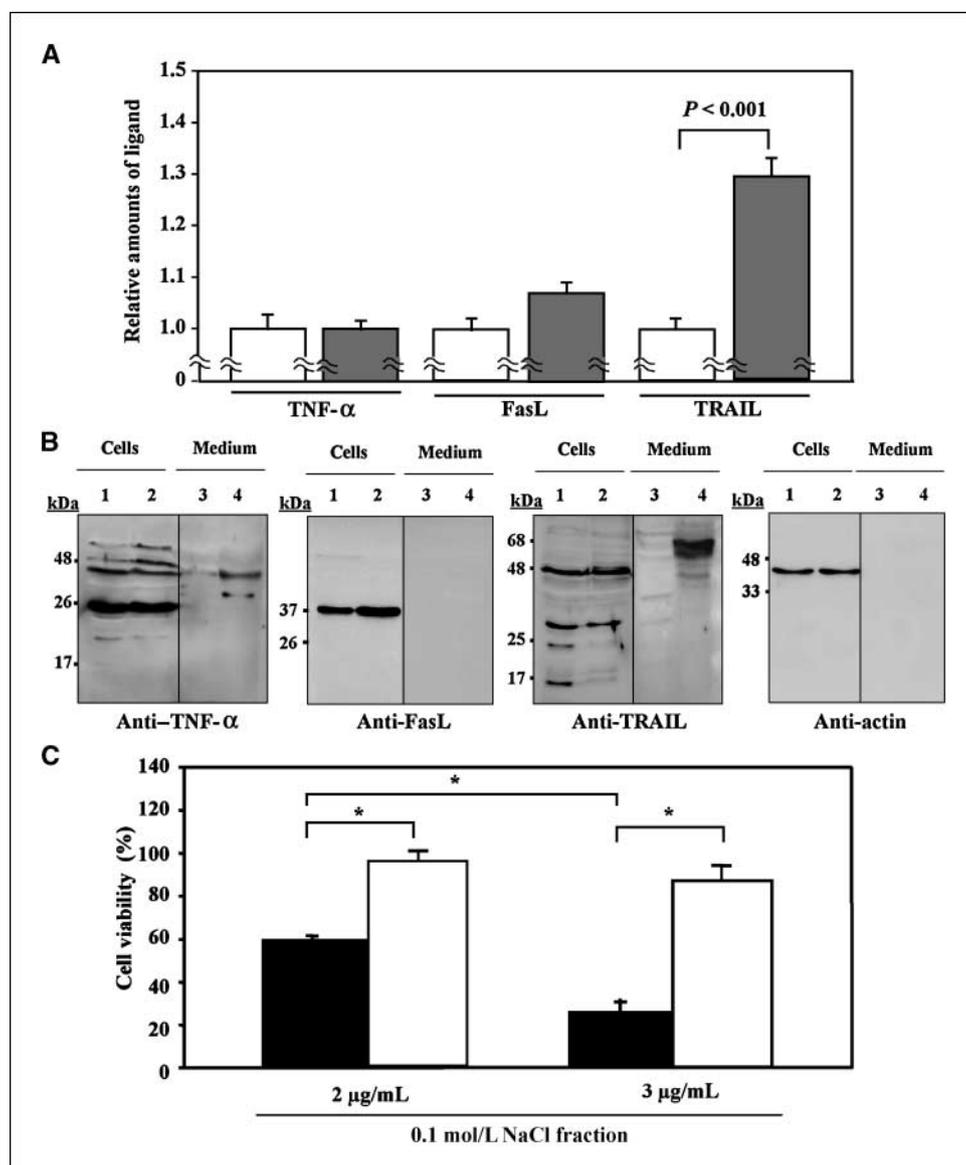
**Susceptibility of various human prostate cancer cell lines to cathepsin E-mediated TRAIL-dependent apoptosis.** We next evaluated the susceptibility of various additional human prostate

cancer cell lines to cathepsin E-induced apoptosis. Whereas cathepsin E had no substantial effect on the viability (Fig. 4A) or morphology (data not shown) of normal PrE cells during incubation for 20 h at 37°C, it significantly reduced the viability of all the cancer cell lines tested without regard to site of origin (ALVA-41, ALVA-101, and PC-3, from bone metastasis; DU145, from brain metastasis; PPC-1, from primary prostate carcinoma),



**Figure 2.** Characterization of cathepsin E-induced apoptosis in tumor cells. *A*, ALVA-41 cells were incubated for 20 h at 37°C and pH 7.4 with the indicated concentrations of cathepsin E purified from rat spleen in the absence (●) or presence (○) of pepstatin A (100 μmol/L). Cell viability was then determined and expressed as a percentage of the value obtained with nontreated cells. Data are means ± SD of values from four independent experiments. \*,  $P < 0.001$ , versus the corresponding value for nontreated cells with pepstatin A. *B*, HEK293 cells were incubated for 20 h at 37°C and pH 7.4 with the indicated concentrations of natural cathepsin E (●), recombinant wild-type (Δ) and its mutant (D98A/D283A; ○). Cell viability was then determined and expressed as a percentage of the value obtained with nontreated cells. Data are means ± SD of values from four independent experiments. \*,  $P < 0.001$ , versus the corresponding value for cells treated with the mutant. *C*, cells were treated with the indicated concentrations of cathepsin E alone as in *A*, after which the culture supernatants were collected, centrifuged to remove debris, and transferred (100 μL) to freshly prepared cell cultures. These cells were then incubated for 20 h at 37°C and pH 7.4 in the absence (●) or presence (○) of pepstatin A (100 μmol/L), after which cell viability was determined and expressed as a percentage of the value of cells treated with vehicle-treated cell culture supernatant. Data are means ± SD of values from four independent experiments. *D*, the culture supernatant of cathepsin E-treated ALVA-41 cells was subjected to DEAE-Sepharose column chromatography as described in Materials and Methods. Cathepsin E (*top*) and cytotoxic activities (*bottom*) of each column fraction were determined with the cathepsin E-specific substrate and freshly prepared ALVA-41 cells, respectively. Cathepsin E activity was expressed as a percentage of the total activity in the culture supernatant; data are means ± SD of values from three independent experiments. The viability of cells treated with each fraction at the indicated final protein concentrations was expressed as a percentage of that of cells treated with vehicle; data are means ± SD of values from four independent experiments.

**Figure 3.** Identification of TRAIL as a mediator of cathepsin E–induced apoptosis in ALVA-41 cells. **A**, after incubation of ALVA-41 cells for 20 h at 37°C and pH 7.4 with cathepsin E (1  $\mu$ mol/L, *gray columns*) or vehicle (*open columns*), the amounts of TNF- $\alpha$ , FasL, and TRAIL in the culture supernatants were determined by ELISAs with specific antibodies to the respective cytokines. Data are expressed relative to the corresponding value for the average of vehicle-treated ones and are the means  $\pm$  SD of values from four independent experiments. **B**, after treatment of the cells with cathepsin E (*lanes 2 and 4*) or vehicle (*lanes 1 and 3*) as in **A**, cell extracts and culture supernatants were subjected to immunoblot analysis with antibodies specific for TNF- $\alpha$  (1/200; Santa Cruz Biotechnology), FasL (1/500; BD Bioscience Pharmingen), TRAIL (diluted 1/300; Stressgen), or  $\beta$ -actin (1/500; Santa Cruz Biotechnology, Inc.) as a loading control. After washing, the membrane was incubated overnight at 4°C with horseradish peroxidase–conjugated secondary antibodies to rabbit IgG (diluted 1/4,000; Biosource International), and then immune complexes were detected with chemiluminescence reagents. **C**, immunodepletion of TRAIL from the 0.1-mol/L NaCl fraction obtained by DEAE-Sephacel chromatography of the culture supernatant of cathepsin E–treated cells at the indicated final protein concentrations with antibodies to TRAIL (*open columns*), but not with control mouse IgG (*black columns*), blocked its apoptosis-inducing activity. Data are expressed as a percentage of the corresponding value for vehicle-treated cell viability and are means  $\pm$  SD of values from three independent experiments. \*,  $P < 0.001$ , versus the corresponding value for cells treated with control IgG.



androgen dependency (dependent, ALVA-41; moderately dependent, ALVA-101; independent, PPC-1, PC-3, and DU145), and extent of differentiation (well, ALVA-41; moderately, DU145; poorly, PPC-1 and PC-3). However, the extent of apoptosis varied among these cell lines, increasing according to the rank order PPC-1 < DU145  $\leq$  ALVA-101 < ALVA-41 < PC-3, with the effect of cathepsin E on the viability of PC-3 cells being about 20 times that apparent for PPC-1 cells. Given that the sensitivity of cells to TRAIL-induced apoptosis is likely dependent on the relative levels of expression of the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) and the decoy receptors TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and osteoprotegerin, we assessed the surface expression of these receptors in these cell lines as well as in PrE cells by SDS-PAGE and immunoblot analysis with antibodies for each molecule. All of the prostate carcinoma cell lines as well as PrE cells similarly expressed not only TRAIL but also all the membrane-associated receptors (DR4, DR5, DcR1, DcR2; Fig. 4B). In contrast, the amount of the soluble decoy receptor osteoprotegerin, which lacks a death domain and prevents the

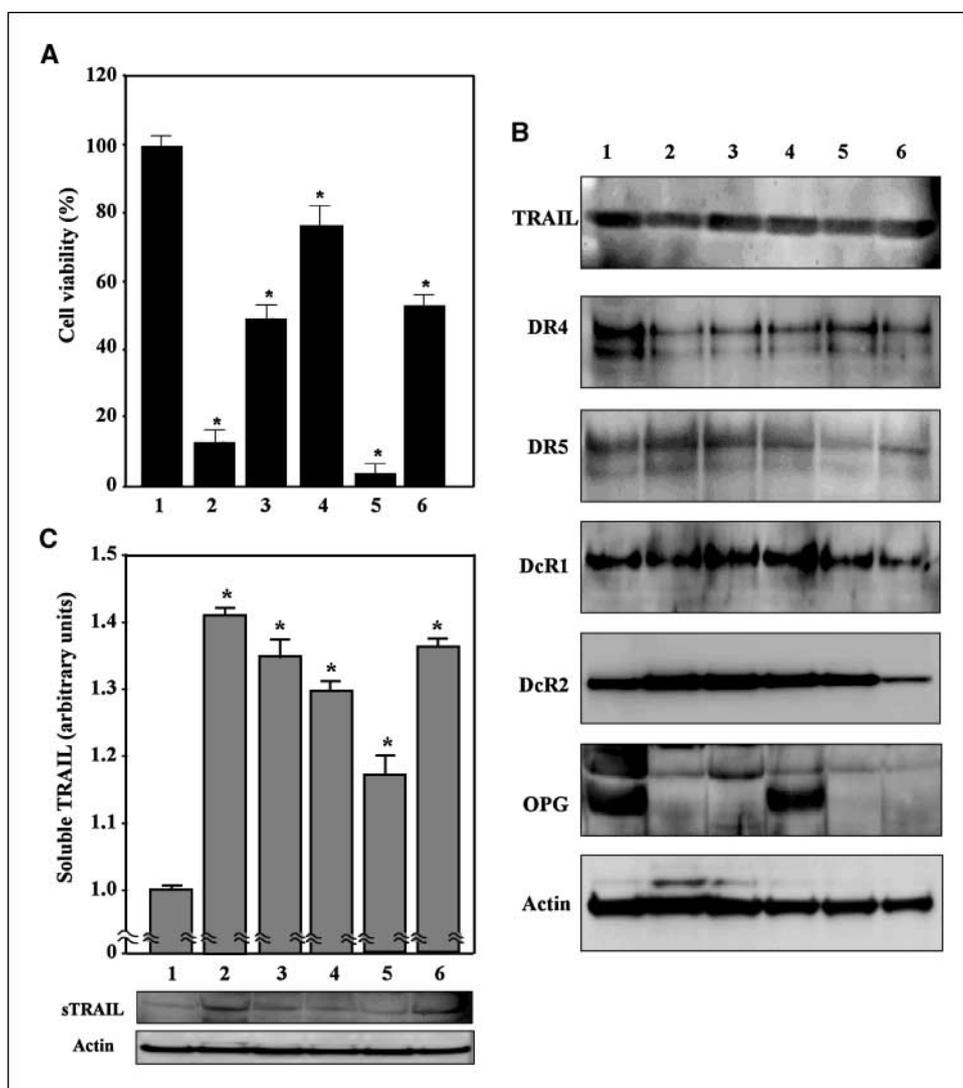
binding of TRAIL to DR4 or DR5, was markedly increased in the culture supernatants of PrE and PPC-1 cells compared with that in those of the other cell lines examined. Given that cancer cell-derived osteoprotegerin is an important survival factor for hormone-resistant prostate cancer cells and that the level of endogenous osteoprotegerin is negatively correlated with the ability of TRAIL to induce apoptosis in prostate carcinoma cells (29), our results suggest that the increased expression of osteoprotegerin may contribute to the relative resistance of PrE and PPC-1 cells to cathepsin E–mediated apoptosis. The amount of soluble trimeric TRAIL released by cathepsin E treatment also differed among the cancer cell lines, increasing according to the rank order PrE < PC-3 < PPC-1 < ALVA-101  $\leq$  DU145 < ALVA-41 (Fig. 4C). Given the similar cell surface expression of TRAIL in all of the cell lines, the difference in the production of soluble TRAIL may be due to a difference in the efficiency of cathepsin E–mediated cleavage of TRAIL at the cell surface. Therefore, increased expression of osteoprotegerin or a reduced efficiency of cathepsin E–mediated cleavage of TRAIL at the cell surface, or

both, may account in part for the difference in susceptibility to cathepsin E-induced apoptosis among the prostate cancer cell lines.

**Suppression of tumor growth and metastasis in nude mice bearing human prostate carcinoma cells by administration of cathepsin E.** To determine whether the anticancer activity of cathepsin E observed *in vitro* was also apparent *in vivo*, we injected purified cathepsin E (2.5 nmol/L/kg) or vehicle once a day for 16 days into the center of tumors formed in nude mice by s.c. implanted ALVA-41 cells. Treatment was initiated when tumors had achieved a volume of about 100 mm<sup>3</sup>. The growth of tumors treated with cathepsin E was markedly inhibited compared with that of tumors treated with vehicle (Fig. 5A). The ability of cathepsin E to induce tumor regression was dose dependent, with obvious benefit at a daily dose of 2.5 nmol/L/kg, reduced benefit at 0.5 nmol/L/kg, and little or no detectable effect at  $\leq 0.25$  nmol/L/kg (data not shown). The mass of xenografts treated with cathepsin E (5 nmol/L/kg) for 10 days after tumor volume had reached  $\sim 400$  mm<sup>3</sup> was significantly smaller than that of those treated with vehicle (Fig. 5B). Importantly, the TUNEL assay revealed that the number of apoptotic cells was markedly increased in tumors treated with cathepsin E compared

with that in tumors treated with vehicle (Fig. 5C), indicating that exogenously administered cathepsin E is effective in inducing apoptosis in tumor cells *in vivo*, like *in vitro*. Given that the proportion of cells undergoing apoptosis in tumors increased with time of treatment, being the highest on the last day of the 16-day regimen, more frequent injections or higher doses of cathepsin E might be more effective at mediating regression of larger preexisting tumors. In contrast, injection of purified cathepsins D or B (5 nmol/L/kg for each) into the center of tumors under the same conditions above did not cause tumor regression (data not shown). Injection of cathepsin E or vehicle into tumors did not result in any observable toxic effects on normal tissues or cells of the treated mice.

**Relationship between endogenous cathepsin E levels and tumor growth reduction.** Several tumor-infiltrating effector cells are known to play an important role in TRAIL-mediated suppression of tumor development or metastasis (29–32). Given preferential expression of cathepsin E in cells of the immune system, including lymphocytes, macrophages, and dendritic cells (4, 33), this enzyme in these cells is more likely to contribute to host defense against tumor cells. We therefore evaluated a potential role of endogenous cathepsin E in the suppression of

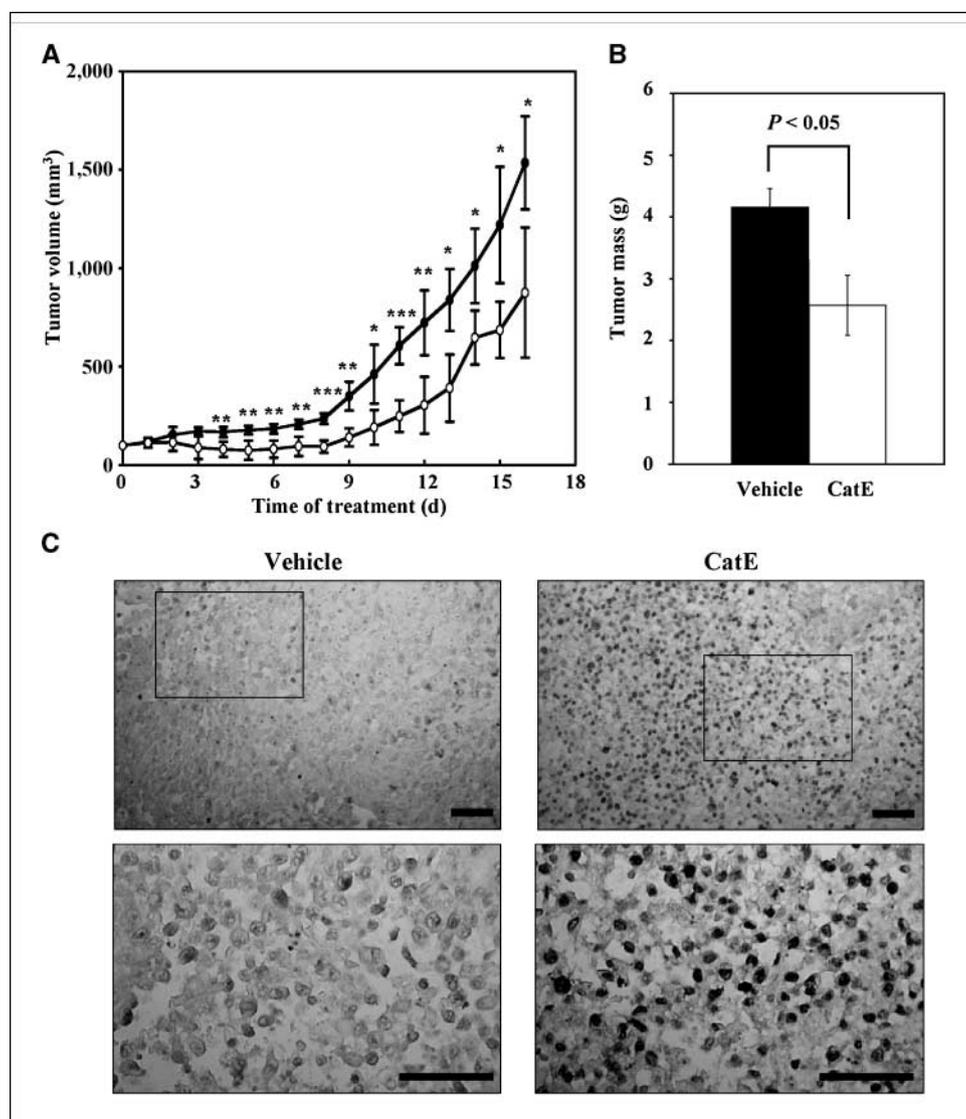


**Figure 4.** Susceptibility of various human prostate carcinoma cell lines to cathepsin E-induced apoptosis. Lane 1, PrE; lane 2, ALVA-41; lane 3, ALVA-101; lane 4, PPC-1; lane 5, PC-3; lane 6, DU145.

\*,  $P < 0.001$ , versus the value for cathepsin E-treated PrE cells. A, various human prostate carcinoma cell lines as well as normal human PrE cells in 10-cm dishes were cultured to  $\sim 80\%$  confluence and washed twice with ice-cold PBS and then labeled with the cell-impermeant, cleavable reagent sulfo-NHS-SS-biotin (Pierce) according to the method as described (50). Then, each cell culture was incubated for 20 h at 37°C and pH 7.4 with cathepsin E (1  $\mu\text{mol/L}$ ) or vehicle. Cell viability was then determined with a colorimetric assay and expressed as a percentage of the corresponding value for vehicle-treated cells. Data are means  $\pm$  SD of values from four independent experiments.

\*,  $P < 0.001$ , versus the corresponding value for vehicle-treated cells. B, cells were biotinylated with the cell-impermeant, cleavable reagent sulfo-NHS-SS-biotin and then lysated, and the cell lysates were incubated with streptavidin-agarose beads for 2 h at 4°C. The beads were then washed, and the associated proteins were subjected to immunoblot analysis with antibodies to TRAIL (diluted 1/300), to DR4 (1/500; BD Bioscience Pharmingen), to DR5 (1/100; R&D Systems), to DcR1 (1/500; Imgenex), to DcR2 (1/500 Imgenex), to osteoprotegerin (1/150; Chemicon), or to  $\beta$ -actin (1/500; Santa Cruz Biotechnology, Inc.). C, culture supernatants of cells treated with cathepsin E as in A were subjected to immunoblot analysis with antibodies to TRAIL or  $\beta$ -actin (bottom). The immunoblots were then subjected to densitometric analysis for quantification of soluble trimeric TRAIL. An arbitrary density unit was defined as the intensity of the TRAIL bands per square millimeter for cathepsin E-treated PrE cells. Data are means  $\pm$  SD of values from four independent experiments.

**Figure 5.** Effects of cathepsin E injection on the growth of tumors formed by ALVA-41 cells in nude mice. **A**, ALVA-41 cells were injected s.c. into male nude mice. When tumor volume reached  $\sim 100 \text{ mm}^3$ , cathepsin E (2.5 nmol/L/kg/d;  $\circ$ ) or vehicle ( $\bullet$ ) was injected into the center of the tumor for 16 d. Data are means  $\pm$  SD of values from five mice per group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$ , versus the corresponding value for cathepsin E-treated mice. **B**, when tumor size reached  $\sim 400 \text{ mm}^3$ , cathepsin E (5 nmol/L in propylene glycol/kg/d) or vehicle (propylene glycol) was injected into the center of each tumor for 10 d, and then tumor mass was determined. Data are means  $\pm$  SD of values from three mice per group. **C**, xenografts of mice treated with cathepsin E or vehicle as in **B** were analyzed by TUNEL assay with a Tumor TACS *In situ* Apoptosis Detection Kit (Trevigen). After reaction with diaminobenzidine, the sections were counterstained with 1% methyl green. Data are representative of results obtained with five mice for each group. *Bottom*, higher magnification views of the images (*squares*) in the top. Bars, 50  $\mu\text{m}$ .



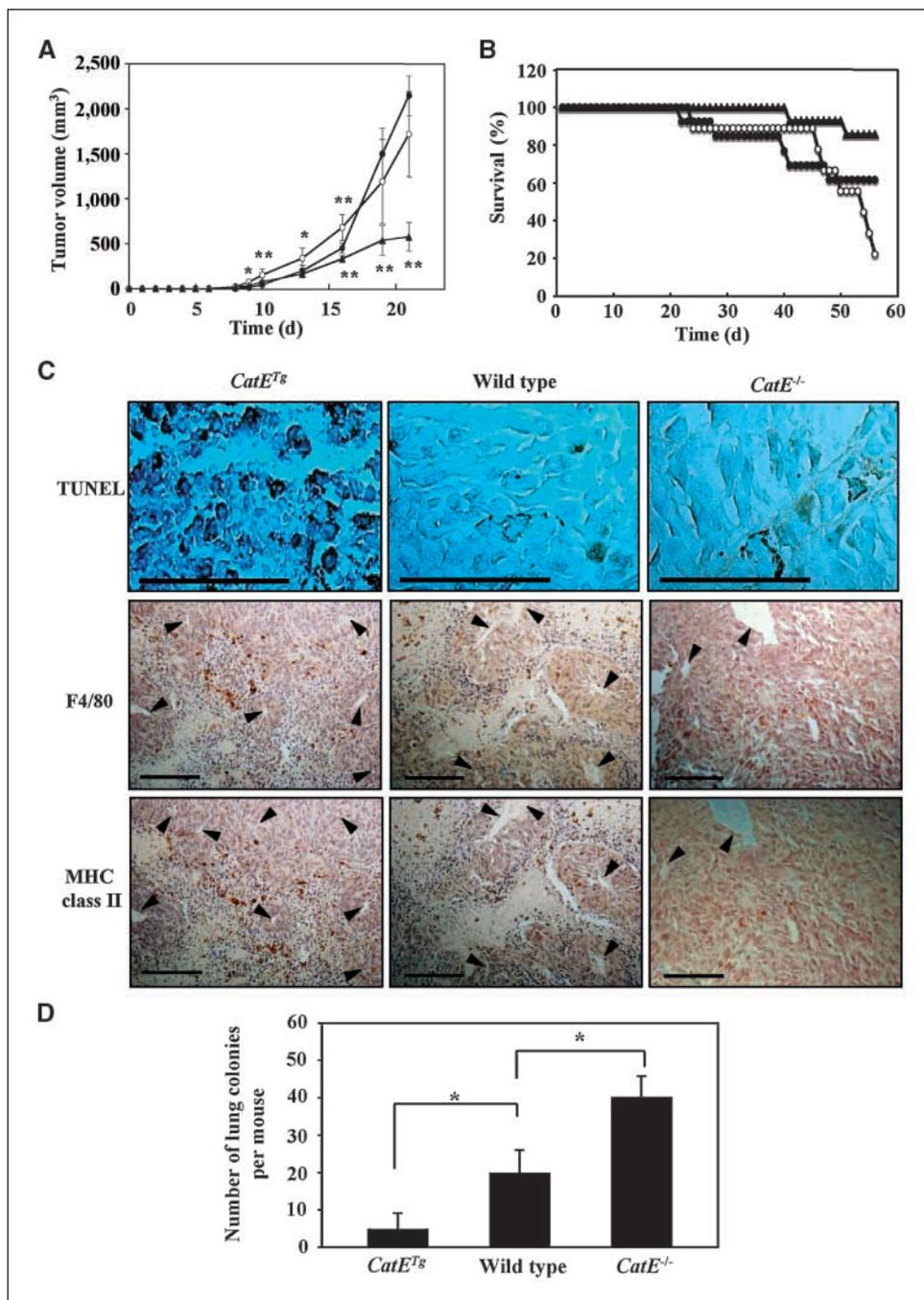
tumor growth, metastasis, and mortality with three different genotypes of syngeneic mice showing different cathepsin E expression levels: wild-type,  $CatE^{-/-}$ , and  $CatE^{Tg}$  mice. Mice of each genotype were injected s.c. with B16 melanoma cells, and then the size of the resulting tumors was monitored for up to 21 days. During the 10 to 15 days postinoculation, the growth of tumors in  $CatE^{-/-}$  mice was significantly higher than that in  $CatE^{Tg}$  mice and wild-type littermates; however, such a difference between wild-type and  $CatE^{-/-}$  mice disappeared at 19 days after inoculation (Fig. 6A), probable due to the outcome of the two concomitantly competing interactions: tumor growth and prevention. Tumor growth was most effectively suppressed in  $CatE^{Tg}$  mice during the whole experimental period, with the value for tumor mass on the last day during the 21 days postinoculation being about one-third and one-fifth those for  $CatE^{-/-}$  mice and wild-type littermates, respectively. We further evaluated the role of endogenous cathepsin E in the survival of mice of each genotype bearing B16 melanoma by monitoring their mortality for 56 days (Fig. 6B). Following inoculation with B16 cells,  $CatE^{-/-}$  mice exhibited increased mortality compared with wild-type and  $CatE^{Tg}$  mice (survival rates of  $CatE^{-/-}$ , wild-type, and  $CatE^{Tg}$  mice

were 20%, 60%, and 80%, respectively). These results indicate that the mortality of tumor-bearing animals is also inversely associated with endogenous cathepsin E levels.

The TUNEL assay of tumor xenografts from each animal group at 23 days postinoculation revealed that the number of apoptotic cells was markedly increased in  $CatE^{Tg}$  mice, whereas only a small amount of TUNEL-positive cells were observed in  $CatE^{-/-}$  mice and wild-type littermates (Fig. 6C, top). Further histologic studies showed that large numbers of tumor-infiltrating effector cells, including lymphocytes and macrophages, were apparent both within and nearby the tumors formed 23 days after the s.c. injection of B16 cells in mice of each genotype. Strikingly, both the number and the extent of activation of infiltrated macrophages were more profound in the tumors of  $CatE^{Tg}$  mice compared with those of wild-type and  $CatE^{-/-}$  mice, as revealed by immunostaining with antibodies to the F4/80 antigen and to MHC class II molecules, respectively (Fig. 6C, middle and bottom). Consistent with these data, previous studies showed an inverse association between the number of macrophages in pleural effusions from cancer patients and the extent of malignant disease (34, 35). It is generally accepted that macrophages, like

lymphocytes, induce tumor lysis through cytotoxic systems, including direct and/or indirect cell killing by reactive oxygen species and reactive nitrogen intermediates, as well as effector molecules such as IFN- $\alpha$ , IFN- $\gamma$ , interleukin-1 (IL-1), and IL-12 (36), besides TRAIL-mediated apoptosis. In addition, activated macrophages effectively kill tumor cells through both antibody-dependent and antibody-independent mechanisms (37, 38). Therefore, apoptosis of B16 melanoma cells and the concomitant tumor growth arrest and the consequent reduction of mortality in *CatE<sup>Tg</sup>* mice are more likely mediated by TRAIL-dependent apoptosis and/or enhanced tumor-infiltrating macrophage-mediated cytotoxicity.

Furthermore, the peripheral necrotic lesion areas of tumors containing dead and dying cells were markedly increased according to the rank order *CatE<sup>Tg</sup>* < wild-type < *CatE<sup>-/-</sup>* mice (Supplementary Fig. S3, top). This was inversely associated with vascular density in these lesion areas, suggesting that the elimination of the dead cells from the tumor area and the infiltration of inflammatory cells into this area through circulation are highly impaired in *CatE<sup>-/-</sup>* mice compared with those in mice of other genotypes. Although the vascularity found in and close to the center of tumors was not significantly different among mice of each genotype, the integrity of vascular endothelial cells in this area was highly vitiated in *CatE<sup>-/-</sup>* mice compared with *CatE<sup>Tg</sup>* mice and wild-type



**Figure 6.** Direct association of endogenous cathepsin E expression levels with the decreased growth and metastasis of mouse B16 melanoma cells in tumors and with the prevention of death of the tumor-bearing mice. *A*, *CatE<sup>-/-</sup>* (○; *n* = 11), wild-type littermates (●; *n* = 10), and *CatE<sup>Tg</sup>* (▲; *n* = 10) were injected s.c. with syngeneic mouse B16 melanoma cells ( $1 \times 10^6$ ), and tumor volume was determined at the indicated times thereafter. Data are means  $\pm$  SD of values from each group. \*, *P* < 0.01; and \*\*, *P* < 0.001, versus the corresponding value for wild-type mice. *B*, mortality of each group after s.c. inoculation with B16 melanoma cells. Difference between the wild-type (●) and *CatE<sup>Tg</sup>* (▲) or *CatE<sup>-/-</sup>* mice (○) was statistically significant. *C*, tumors from mice of each genotype s.c. inoculated with B16 melanoma cells were analyzed by TUNEL assay with a Tumor TACS *In situ* Apoptosis Detection Kit. After reaction with diaminobenzidine, the sections were counterstained with 1% methyl green. Data are representative of results obtained with five mice for each group at 23 d. Top, bars, 100  $\mu$ m. Immunohistochemical staining of tumors from each animal group at 23 d with antibodies to the F4/80 antigen (middle) and to MHC class II molecules (bottom). Arrowheads, blood vessels. Bars, 100  $\mu$ m. *D*, each animal group (*n* = 8 for *CatE<sup>Tg</sup>*, *n* = 12 for wild-type, and *n* = 6 for *CatE<sup>-/-</sup>*) was injected i.v. with B16 melanoma cells ( $2 \times 10^5$ ) and killed 22 days later for the determination of the number of black nodules on the lung surface. Data are means  $\pm$  SD of values from each group. \*, *P* < 0.01, versus the values for the corresponding mice.

littermates (Supplementary Fig. S3, *bottom*). Such a rudimentary structure of the vascular endothelial cell layer in *CatE*<sup>-/-</sup> mice may facilitate leakage of the vascular contents. Finally, to test whether endogenous cathepsin E levels affect the extent of metastasis, B16 melanoma cells were injected i.v. into mice of each genotype, and the number of metastatic colonies in the lungs was determined 22 days later. The number of such colonies in *CatE*<sup>-/-</sup> mice was about 2- and 8-fold that in the wild-type controls and *CatE*<sup>Tg</sup> mice, respectively (Fig. 6D), indicating that cathepsin E deficiency promoted lung metastasis.

## Discussion

In the present study, we provide the first evidence that cathepsin E is responsible for specific cleavage of TRAIL at the surface of tumor cells and the consequent generation of a soluble trimeric form of this protein and thereby induces the growth arrest and apoptosis in the tumor cells without affecting normal cells. Although the culture media of all of the cathepsin E-treated carcinoma cells induced apoptosis in the tumor cells, their apoptosis-inducing potentials varied among the cell lines. This could be explained in part by differences in the expression of the soluble decoy receptor osteoprotegerin or by the efficiency of cathepsin E-mediated cleavage of TRAIL at the cell surface, or both. However, the highest sensitivity of PC-3 cells to cathepsin E-induced apoptosis cannot be explained by either of these two mechanisms. We thus speculate that additional mechanisms exist for the determination of the susceptibility of tumor cells to cathepsin E-induced apoptosis. It is possible that the expression of antiapoptotic proteins such as FLIP, IAPs, Bcl-x<sub>L</sub>, or Bcl-2 may be reduced in PC-3 cells compared with that in the other cell lines.

The antitumor activity of cathepsin E apparent *in vitro* was also corroborated by *in vivo* studies with mice bearing human or mouse xenografts. Here, we showed that daily treatment of nude mice bearing human prostate carcinoma xenografts with cathepsin E induced tumor growth arrest and apoptosis in tumor cells without any apparent histologic effects on normal tissues and cells. Given that several tumor effector cells including activated T cells (33, 39), B cells (40), natural killer cells (41), dendritic cells (32), and monocytes (30, 42) are known to produce TRAIL, cathepsin E injected into mice may exert tumoricidal activity by the generation of soluble TRAIL from these immune system cells, as well as from tumor cells. Furthermore, it is interesting to note that apoptosis of B16 melanoma cells in *CatE*<sup>Tg</sup> mice may be mediated by additional TRAIL-independent mechanisms, because these cells are known to be resistant to TRAIL-induced apoptosis (31, 43). Indeed, *in vitro* studies revealed that B16 cells used were more resistant to cathepsin E-mediated TRAIL-dependent apoptosis than ALVA-41 cells (80% versus 10% resistance). Importantly, however, not only the number of apoptotic B16 melanoma cells, but also the density of tumor-infiltrated effector cells, particularly activated macrophages, in the tumor site was more profound in *CatE*<sup>Tg</sup> mice compared with that in wild-type littermates and *CatE*<sup>-/-</sup> mice. Tumor cells are known to be eliminated by such tumor-infiltrating effector cells through several kinds of mechanisms: killing by phagocytosis, antigen processing and presentation to T4 lymphocytes, and enhanced secretion of various cytokines that play crucial roles in nonspecific host defense (28, 44, 45). In the tumor microenvironment, therefore, it is more likely that multiple mechanisms including TRAIL-dependent apoptosis and tumor-

associated macrophage-mediated cytotoxicity could participate in host defense against tumor cells. The present results clearly showed that tumors formed by B16 cells in *CatE*<sup>-/-</sup> mice contained fewer infiltrating macrophages, and that the activation status of these cells was reduced compared with those in *CatE*<sup>Tg</sup> mice and even in wild-type littermates. Given the strong association of cathepsin E with the activation and functions of macrophages (46, 10) and the positive correlation of IFN stimulation of macrophages with the enhanced expression of TRAIL and the increased killing activity against cancer cells (30), cathepsin E is more likely to exert antitumor activity via not only TRAIL-dependent apoptosis but also tumor-infiltrated activated macrophage-mediated cytotoxicity. In contrast to their anticancer effects through their manifold functions, many deleterious functions of tumor-infiltrated macrophages have also been recently recognized, such as enhancement of tumor cell migration and invasion, facilitation of extracellular matrix breakdown and remodeling, promotion of tumor cell motility, and stimulation of angiogenesis (47–49). These competing functions seem to arise from the pleiotropic nature of the macrophages, which may result from the production of a variety of cytokines and reactive oxygen species by these cells. In this study, we showed the positive association of the growth arrest and metastasis reduction of B16 melanoma cells with endogenous cathepsin E levels and the concomitant increase of tumor-infiltrated activated macrophages, thus suggesting that these cells eradicate the tumor cells through cytotoxicity and apoptosis. Our data also suggest that cathepsin E may contribute to the regulation of the vascular system in tumors via tumor-infiltrated activated macrophages because tumor-associated macrophages have been shown to play a role both in the initiation of angiogenesis in avascular areas and in the remodeling of the vasculature once formed to give coherent vascular flow (48, 49). Indeed, the vascular density in the peripheral necrotic lesion areas of tumors from *CatE*<sup>Tg</sup> mice was significantly higher than that from wild-type and *CatE*<sup>-/-</sup> mice. The integrity of vascular endothelial cells in the center of tumors was highly vitiated in *CatE*<sup>-/-</sup> mice compared with *CatE*<sup>Tg</sup> mice and wild-type littermates. Therefore, tumor cells are more likely to be recognized, destroyed, and eliminated through beneficial functions of tumor-infiltrated activated macrophages rather than their deleterious functions.

A common hurdle that almost all of the anticancer drugs have had not overcome is their severe side effects. Targeting apoptosis in tumor cells without any cytotoxic effect on normal cells is a promising strategy for cancer drug discovery. The present findings suggest that the administration of cathepsin E or agents capable of increasing endogenous cathepsin E expression may be a promising strategy for antitumor therapy. Therefore, cathepsin E-based tumor therapy may be used either alone or in combination with other therapeutic strategies in the treatment of human cancer *in situ*. Studies are now in progress to evaluate the potential beneficial effects of cathepsin E in combination with conventional chemotherapies in the treatment of cancer *in vivo*.

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## Cathepsin E Prevents Tumor Growth and Metastasis by Catalyzing the Proteolytic Release of Soluble TRAIL from Tumor Cell Surface

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