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The Catalytically Inactive Precursor of Cathepsin D Induces Apoptosis in Human Fibroblasts and HeLa Cells

Olga Schestkowa,[1] Dominik Geisel,[1] Ralf Jacob,[2] and Andrej Hasilik[1]*

[1]Department of Physiological Chemistry, Philipps-University Marburg, Marburg, Germany
[2]Cell Biology and Cell Pathology, Philipps-University Marburg, Marburg, Germany

Abstract In several reports cathepsin D has been implicated in apoptosis. In some systems the effects of agents considered to be mediated by cathepsin D were inhibited in the presence of pepstatin A, an inhibitor of the enzyme. In other studies the effect of a mutant cathepsin D deprived of activity was indistinguishable from that of the normal enzyme. Here we show that in human fibroblasts and in HeLa cells apoptosis can be induced by microinjecting into cytosol either mature cathepsin D or its inactive precursor procathepsin D. The microinjected precursor remains in the uncleaved form. These results confirm that the proapoptotic effect of cathepsin D in the cytosol is independent of its catalytic activity and suggest that the interaction of cathepsin D with the downstream effector does not involve the active site of the enzyme, since in the proenzyme the active site is masked by the prosequence. J. Cell. Biochem. 101: 1558–1566, 2007.
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Key words: cathepsin D; apoptosis; procathepsin D; microinjection

In the last two decades, the phenomenon of apoptosis has attracted great interest. In the course of the programmed cell death, several proteinases and endonucleases become activated. The class of proteinases known as caspases play an essential role in the induction and execution of apoptosis. After activation, they become competent to cleave a set of intracellular substrates, which results in biochemical and morphological changes associated with apoptosis [Cohen, 1997].

A number of different signals were identified that activate caspases through complex pathways. These involve nearly all subcellular compartments including lysosomes. Cathepsins D, B, and L have been implicated in apoptosis in a number of cell systems [Deiss et al., 1996; Ishisaka et al., 1998, 1999; Guicciardi et al., 2000; Yeung et al., 2006]. The role of cathepsin D was contemplated from reports on inhibition of apoptosis in the presence of pepstatin A [Roberg et al., 2002].

Cathepsin D is a ubiquitous lysosomal aspartic endoproteinase that plays an important role in protein degradation and generation of bioactive proteins [Barrett, 1970]. It is synthesized as an inactive precursor [Hasilik et al., 1982]. The Precursor is a 53 kDa protein that is proteinolytically processed to a mature enzyme consisting of small and large mature chains of 14 and 31 kDa [Hasilik and Neufeld, 1980]. Roberg et al. [2002] showed that by microinjecting cathepsin D into the cytosol of human fibroblasts, apoptosis is induced in the absence but not in the presence of pepstatin A.

It was shown that cathepsin D mediates cytochrome c release and caspase activation in stauroporine-induced apoptosis in human fibroblasts [Johansson et al., 2003]. Cathepsin D is a key mediator of IFN-gamma-, Fas/APO- and, TNF-alpha-induced apoptosis [Deiss et al., 1996]. Furthermore it is released into the cytosol during apoptosis caused by oxidative stress [Roberg et al., 1999; Ollinger, 2000; Kågedal et al., 2001]. Its activity is enhanced during adriamycin induced apoptosis [Wu et al., 1998]. One of the target molecules of cathepsin D is thioredoxin-1 that was proposed to be degraded by this cathepsin during apoptosis

*Correspondence to: Andrej Hasilik, Department of Physiological Chemistry, Philipps-University Marburg, Karl-von-Frisch-Straße 1, 35043 Marburg, Germany.
E-mail: hasilika@staff.uni-marburg.de

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[Haendeler et al., 2005]. Overexpression of cathepsin D was shown to enhance chemosensitivity and apoptotic response to etoposide [Beaujouin et al., 2006]. Cathepsin D was described to trigger an activation of the Bax protein [Bidere et al., 2003]. In another study, the Bid protein was presented as a direct downstream target of cathepsin D, and the ability of cathepsin D to cleave it in vitro was demonstrated [Heinrich et al., 2004].

In various models of apoptosis pepstatin A, an inhibitor of cathepsin D, suppressed apoptosis [Roberg et al., 1999, 2002; Ollinger, 2000; Kågedal et al., 2001]. However, other reports indicated that the proteinolytic activity of cathepsin D is dispensable for at least a part of its proapoptotic effects [Tardy et al., 2003; Beaujouin et al., 2006]. Hence, the role of the catalytic activity of cathepsin D in apoptosis is still unclear. This suggested to examine the effects of cytosolically microinjected procathepsin D.

MATERIALS AND METHODS

Cell Culture

Human foreskin fibroblasts (NHDF 1C, passages 8–15, Promocell, Heidelberg, Germany) and HeLa cells were cultured in Eagle's Minimum Essential Medium or Dulbecco's Modified Eagle's Medium supplemented with 2 mmol/L glutamine, 50 IU/ml penicillin-G, 50 g/ml streptomycin, and 10% fetal bovine serum. Twenty-four hours before the experiments, the cells were trypsinized, counted, and seeded at a density of 10,000 cells/cm^2 on cover slips [Roberg et al., 2002].

Microinjection

Microinjection was performed on the stage of a Zeiss Axiovert 10 (Zeiss, Jena, Germany) inverted microscope, using a pressure injector (Model 5247-01643; Eppendorf, Hamburg, Germany) and an Injectman NI-2 micromanipulator (Eppendorf). Eppendorf microloaders were used to fill the microinjection needles (Femtotips II, Eppendorf), that had an inner diameter of less than 0.5 μm. All injectates contained 1 mg/ml dextran-conjugated Alexa Fluor 488 or 0.3% dextran-conjugated Texas Red Dye (both MW 10,000, Molecular Probes, Eugene, Oregon) in PBS (Dulbecco's phosphate-buffered saline pH 7.0), which was used as the vehicle control. Freshly prepared Alexa Fluor 488 containing 0.5 mg/ml procathepsin D (biosynthesis in Spodoptera cells and purification were performed as described previously [Grosch and Hasilik, 1998; Gopalakrishnan et al., 2004]), 0.5 mg/ml cathepsin D [Roberg et al., 2002], purified from human trophoblastic tissue [Hasilik and Neufeld, 1980], 3 mg/ml of cytochrome c [Li et al., 1997] (C 7752, Sigma) were injected into the cytoplasm of human fibroblasts and HeLa cells with a pressure of 100 hPa during a period of 1.2 s. These parameters were kept constant in all experiments, independent of the microinjectate contents. Caspase-3-like proteinase inhibitor Ac-DEVD-CHO (235420, Calbiochem, San Diego), pepstatin A (Sigma) and prosaposin were microinjected at concentrations of 25, 5, and 9 μM respectively. C_2-ceramide (N-acetyl-D-sphingosine, A7191, Sigma) and saposin B (obtained from Dr. S. Locatelli-Hoops, University of Bonn) dissolved in PBS were microinjected at concentrations of 13 μM dissolved in PBS with 1% DMSO, which was used as vehicle control in these experiments. In each dish 100–300 cells were injected. The results are presented as average values for at least four dishes.

Western Blot Analysis

To examine the possibility of an activation of the microinjected procathepsin D by a proteinolytic cleavage 6,000 cells were injected with a solution of the protein and the vehicle control and harvested after 8 h incubation. The cells were lysed using 20 μl solubilizing mixture (20 mM DTT, 2% w/w SDS, 0.5 M Tris pH 6.8, 26% v/v glycerol). For Western blot analysis, samples were subjected to 10% SDS–PAGE and proteins were electroblotted onto a nitrocellulose membrane. The blots were incubated for 30 min with 5% dried skimmed-milk powder dissolved in 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM $CaCl_2$, 0.01% antifoam A, 0.05% Triton X-100, 0.02% sodium azide (blocking buffer) and overnight with rabbit anti-cathepsin D antibody diluted 1:3,000 in 10 ml of blocking buffer, with continuous agitation. The blots were washed for 30 min in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) and TBST (TBS containing 0.5% Tween-20) and then incubated for 2 h at 37°C with goat anti-rabbit IgG-HRP conjugate (BioRad, Herts, UK) diluted 1:6,000 in the blocking buffer. After a washing step with TBS and TBST, the ECL™ Western Blotting Detection Reagents

(Amersham Pharmacia Biotech) were used following the instructions of the manufacturer. After 2 min incubation time the blots were exposed to X-ray film (Kodak X-OMAT) for 5 or 15 min.

Detection of Apoptosis and Statistical Analysis

After microinjecting the cells varied solutions, the medium was replaced and following a 30 min incubation at 37°C and 5% CO_2, the cells were photographed using BX-61 fluorescence microscope + BX-UCB, U-HSTR-2, U-RFL-T (Olympus, Hamburg, Germany). The washing was repeated and the cells were incubated in fresh medium as above for 11.5 h. After the total incubation period of 12 h 4 μg/ml 4,6-diamidino-2-phenylindole, DAPI (Hoechst Dye 33342, Sigma) was added to the medium and the incubation was continued for 30 min to stain the nuclei. Afterwards, the cells were washed and photographed again. The fluorescent cells that were rounded and shrunken and contained pycnotic nuclei were considered as apoptotic. The percentage of the apoptotic cells was statistically analyzed using the Mann–Whitney *U*-test. *P*-values <0.05 were considered significant.

Detection of Phosphatidylserine Translocation From the Inner to the Outer Plasma Membrane Leaflet

After the incubation period the cells were washed in cold PBS and resuspended in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) [Koopman et al., 1994]. Annexin V Alexa Fluor 350 conjugate (Molecular Probes) 5–25 μl and 1.5 μM propidium iodide were added to each 100 μl annexin-binding buffer and the cells were incubated at room temperature for 15 min. After a washing step with annexin-binding buffer the staining was examined by fluorescent microscopy.

Detection of DNA Fragmentation

Additionally, occurrence of apoptosis was verified using the Apoptosis ELISA Plus Kit (Roche Diagnostics GmbH, Mannheim, Germany). Approximately 2,000 cells were injected and lysed 6–12 h later using the lysis buffer of the kit. The ELISA was performed according to the instructions of the manufacturer.

RESULTS

Microinjection of Procathepsin D Induces Apoptotic Morphology

We observed that microinjection of 0.5 mg/ml solution of procathepsin D into the cytoplasm of human cultured fibroblasts induced apoptotic morphology. In similar experiments (not shown) it was observed that the proportion of apoptotic cells did not increase at later time points. Staining with DAPI revealed pycnotic nuclei (Fig. 1). Staining with Annexin V Alexa Fluor 350 conjugate indicated a translocation of phospatidylserine from the inner to the outer leaflet of the plasma membrane, a characteristic of early apoptosis (Fig. 2). In cells microinjected with cathepsin D the rate of apoptosis reached a plateau at 12 h after microinjection when 46% cells were affected (Fig. 3).

This latter result reproduced the findings by Roberg et al. [2002]. We also microinjected cytochrome c at the concentration 3 mg/ml as reported by Li et al. [1997] to induce apoptosis in Human 293 kidney cells and HeLa cells. Using the same pressure and time as for microinjection of procathepsin D, we found an apoptotic rate of 60% after 12 h of incubation. In control experiments the apoptotic morphology reached 7% after 12 h (Fig. 3).

To illustrate the mean effects of the different microinjections such as shrinkage of individual cells, we took fluorescent microscopy photographs of large cell-collectives at 30 min and 12 h after the microinjection (Fig. 4). Microinjections of the three proteins were also performed with HeLa cells and similar results were obtained (Fig. 5).

Inhibition of Caspase 3 Prevents Procathepsin D-Induced Apoptosis

Caspase 3 is one of the most important executioners of apoptosis, being responsible for the proteinolytic cleavage of key proteins in programmed cell death [Cohen, 1997]. It was shown that cathepsin D mediates cytochrome c release and afterwards activation of caspases [Brustugun et al., 1998; Kågedal et al., 2001; Johansson et al., 2003].

To explore a participation of the caspase cascade in the apoptosis induced by procathepsin D, we microinjected 1,912 cells with 25 μM caspase-3-like proteinase inhibitor Ac-DEVD-CHO in combination with 0.5 mg/ml procathepsin D. As compared to the control,

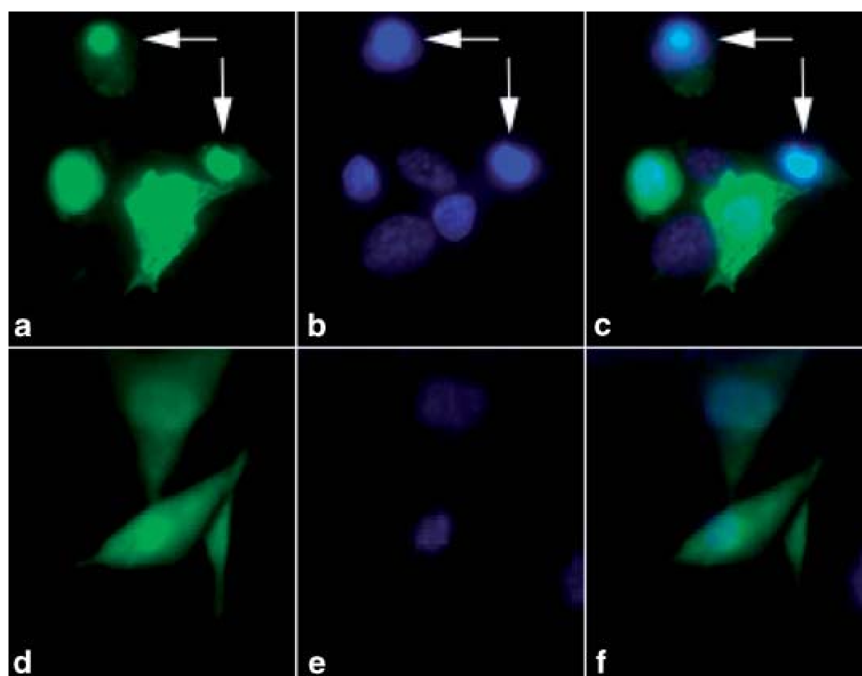

Fig. 1. Apoptosis of human fibroblasts after microinjection of 1 mg/ml Alexa Fluor in PBS pH 7.0 with 0.5 mg/ml procathepsin D (upper row) or Alexa Fluor in PBS pH 7.0 alone (lower row). Apoptotic cells were detected by DAPI staining and shrunken morphology, examples are indicated by arrows. **a**–**c**: Cells microinjected with 1 mg/ml Alexa Fluor in PBS pH 7.0 in conjunction with 0.5 mg/ml of procathepsin D. **d**–**f**: Cells microinjected with 1 mg/ml Alexa Fluor in PBS pH 7.0 (which was previously used as vehicle control). Using 100 hPa and 1.2 s as microinjection parameters in all experiments we could obtain the same total microinjection volume in all samples. The microinjected cells were visualized with Alexa Fluor 488 (green fluorescence) and the nuclear morphology with 4,6-diamidino-2-phenylindole (blue fluorescence).

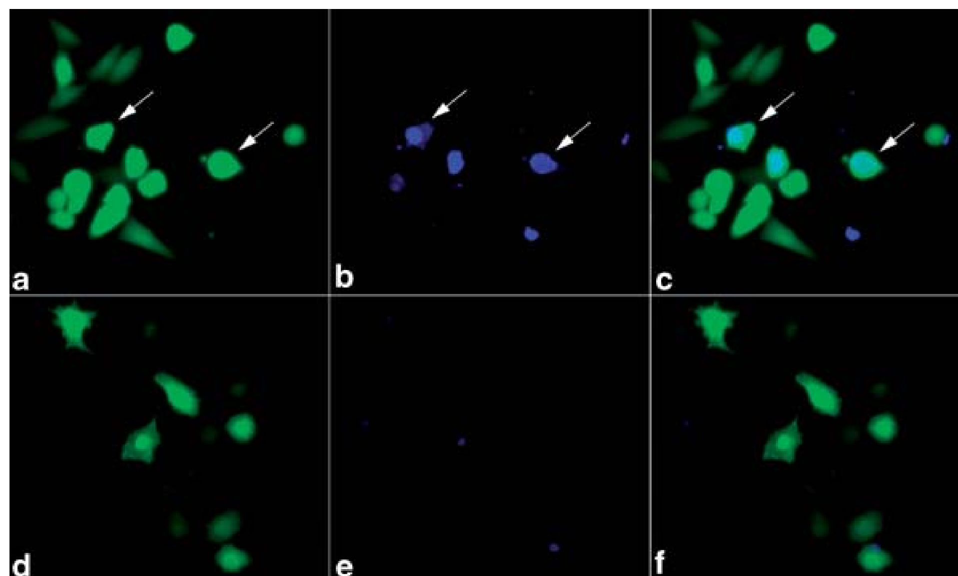

Fig. 2. Apoptosis of HeLa cells after microinjection of 1 mg/ml Alexa Fluor in PBS pH 7.0 and 0.5 mg/ml procathepsin D. Apoptotic cells were detected by Annexin V Alexa Fluor 350 conjugate, which detects the translocation of phosphatidylserine from the inner to the outer plasma membrane leaflet in early stages of apoptosis. Apoptotic HeLa cells show blue fluorescence beside green fluorescence caused by Alexa Fluor 488 microinjection. Examples are indicated by arrows. **a**–**c**: Cells microinjected with 1 mg/ml Alexa Fluor in PBS pH 7.0 in conjunction with 0.5 mg/ml of procathepsin D; a: Alexa Fluor staining; b: Annexin V staining, c: an overlay of a and b. **d**–**f**: Cells microinjected with 1 mg/ml Alexa Fluor in PBS pH 7.0 (which was previously used as vehicle control); d: Alexa Fluor staining; e: Annexin V staining; f: an overlay of d and e.

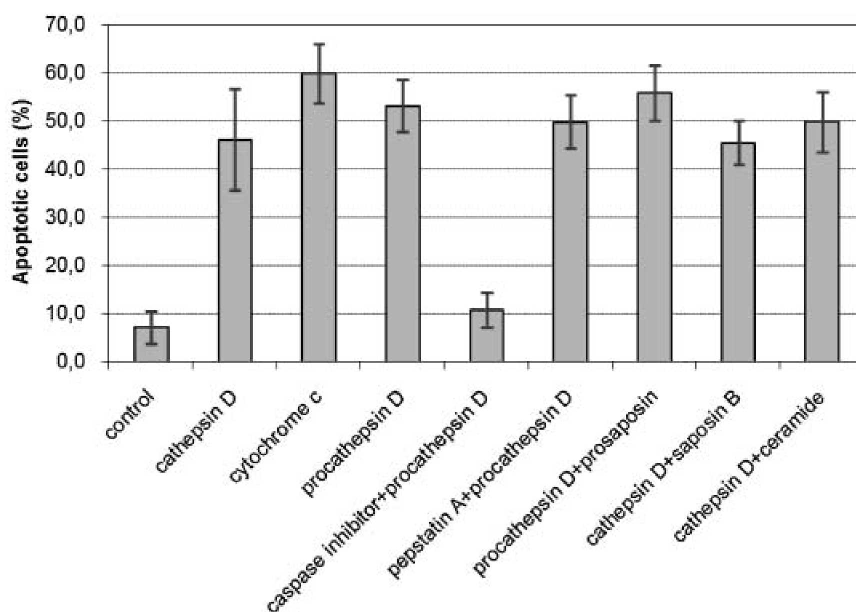

Fig. 3. Apoptosis 12 h after microinjection. The fibroblasts were microinjected with 1 mg/ml Alexa Fluor PBS pH 7.0 alone or together with 3 mg/ml cytochrome c, 0.5 mg/ml of cathepsin D and procathepsin D together or without 5 μM pepstatin A; 25 μM Ac-DEVDCHO, 9 μM prosaposin, 13 μMol C2-ceramide, 13 μM saposin B. Values represent mean percentages and standard deviations of the apoptotic cell counts from at least four separate experiments.

apoptosis was not increased after this treatment (Fig. 3). When a mixture of cathepsin D and cytochrome c was supplemented with the caspase 3 inhibitor, the apoptosis was prevented, too. Also in HeLa cells a strong inhibition of procathepsin D-induced apoptosis was observed (Fig. 5).

Pepstatin A Does Not Prevent Procathepsin D-Induced Apoptosis

The induction of apoptotic morphology in 800 cells by procathepsin D was not prevented by including 5 μM pepstatin A. The observed percentage of apoptotic cells after 12 h was 54% (Fig. 3) and, thus, similar to cells treated with procathepsin D alone. In a control experiment cathepsin D was microinjected into 500 cells in a combination with pepstatin A (not shown). After 12 h 45% apoptotic cells were observed.

Furthermore, a combined microinjection of procathepsin D and prosaposin resulted in 56% apoptosis after 12 h (Fig. 3). Microinjections of cathepsin D with either C_2-ceramide or saposin B did not show a higher rate of apoptosis than those of cathepsin D alone (Fig. 3).

Microinjected Procathepsin D Is Not Processed in the Cytoplasm

To examine possible processing of procathepsin after its microinjection into the cytoplasm of human fibroblasts, a Western blot analysis was performed using anti-cathepsin D antibodies. For this purpose, 6,000 cells were microinjected with 0.5 mg/ml procathepsin D. The microinjected procathepsin D remained unchanged in the cytoplasm for at least 8 h by the criteria of the apparent molecular weight (Fig. 6). We conclude that a cleavage of procathepsin D is not taking place during the observation period.

DISCUSSION

The purpose of our study was to examine the role of the proteinolytic activity of cathepsin D in induction of apoptosis. As a model of enzymatically inactive cathepsin D we used procathepsin D. Our results show that a cytosolic delivery of procathepsin D results in morphologically similar apoptosis as compared to cathepsin D. We observed that procathepsin D was not processed to its active forms in cytosol. In the presence of procathepsin D and a caspase-3-like inhibitor, the apoptotic rate after 12 h was

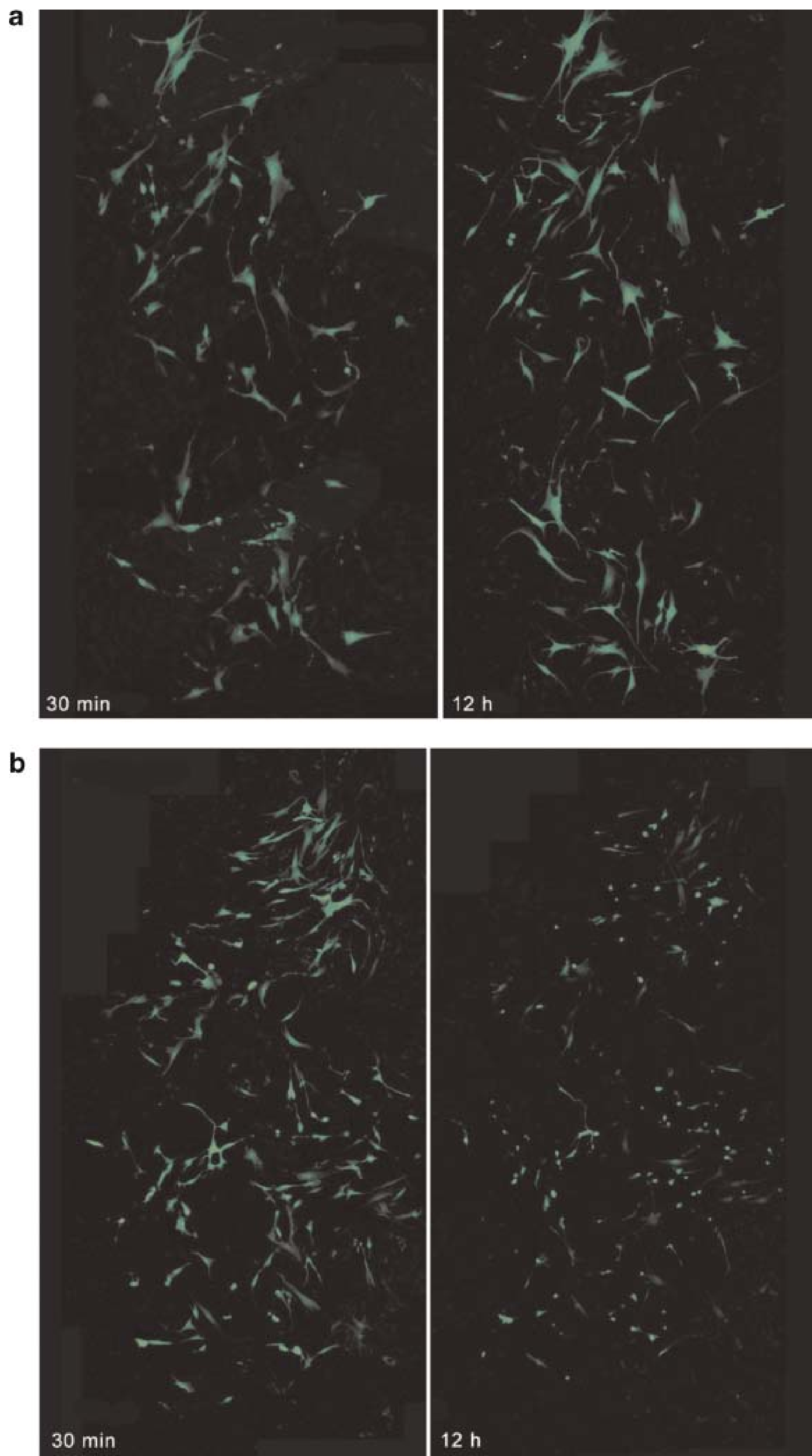

Fig. 4.

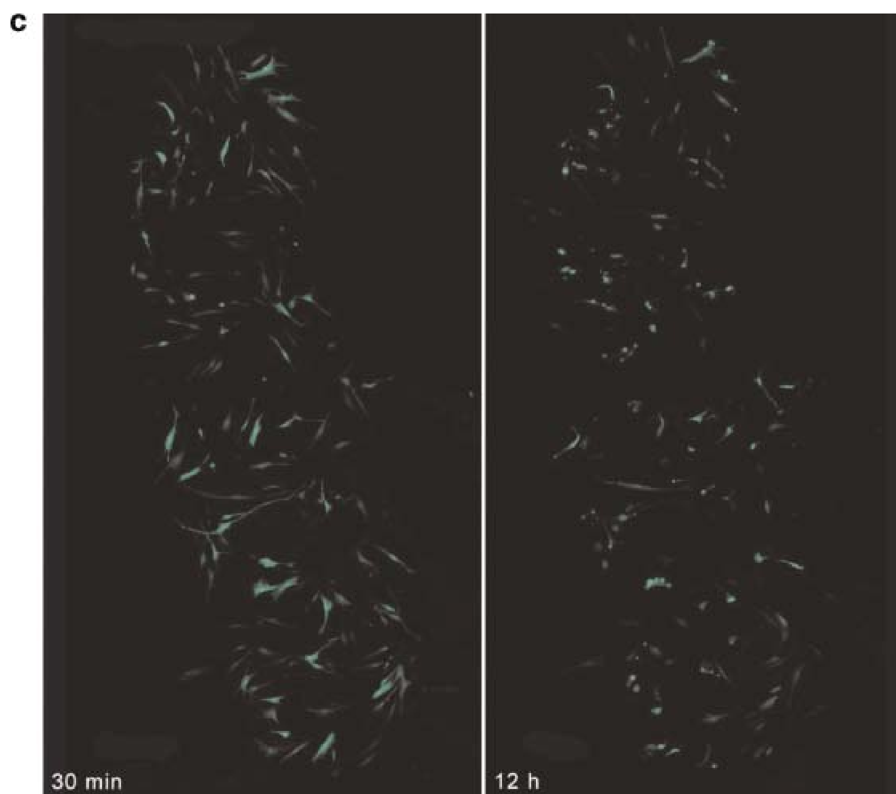

Fig. 4. Apoptosis after microinjection into the cytosol of human fibroblasts. A comparison of changes in cell morphology between 30 min and 12 h post-injection. A 470–490 nm filter was used to visualize Alexa Fluor 488 distribution in the cytoplasm. **a**: Controls microinjected with 1 mg/ml Alexa Fluor in PBS pH 7.0. **b**: Microinjection of 1 mg/ml Alexa Fluor in PBS pH 7.0 and 0.5 mg/ml cathepsin D. **c**: microinjection of 1 mg/ml Alexa Fluor in PBS pH 7.0 and 0.5 mg/ml procathepsin D. In **panels b–c** a cell shrinkage was observed after 12 h incubation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

comparable to the apoptotic rate in the control. We suggest that procathepsin D induces apoptosis by the same cytosolic interactions as cathepsin D. Thus, we propose that the yet unidentified target of cathepsin D is unlikely to be cleaved by cathepsin D. It appears to bind both procathepsin D and cathepsin D and the complexes seem to be equivalent in inducing apoptosis. The interaction is likely not to involve the substrate binding site of the cathepsin D molecule since in the proenzyme this site is covered by the prosequence moiety.

The present hypothesis is further affirmed by results with pepstatin A. This inhibitor of cathepsin D does not affect apoptosis induced by either cathepsin D or procathepsin D in both fibroblasts (Fig. 3) and HeLa cells (Fig. 5). Roberg et al. [2002] have reported on an inhibition of the apoptosis after microinjecting cathepsin D that had been preincubated with pepstatin A. Since pepstatin A is known to bind the active site of cathepsin D it can be considered to inhibit binding or to prevent the cleavage of the possible targets. However, this explanation is unlikely, because the enzymatic activity and the binding of pepstatin A both depend on acidic pH. Furthermore, we could not prevent apoptosis by adding pepstatin A to cathepsin D before microinjection in 500 fibroblasts.

Most of the present results were obtained with two different cell lines, primary human fibroblasts and the cervix cancer cell line HeLa. Therefore, we conclude that our concept of apoptosis induction with procathepsin D is not a specific feature of one cell line and plays a role in cancer and primary cell lines.

Our results confirm the proapoptotic effect of microinjected cathepsin D [Roberg et al., 2002]. However, in our system we could not reproduce the inhibition of this effect by pepstatin A. Haendeler et al. [2005], reported on a catalytical action of cathepsin D degrading thioredoxin-1 into apoptosis inducing metabolites but without translocation of the enzyme into the cytosol. Beaujouin et al. [2006] observed that wild-type

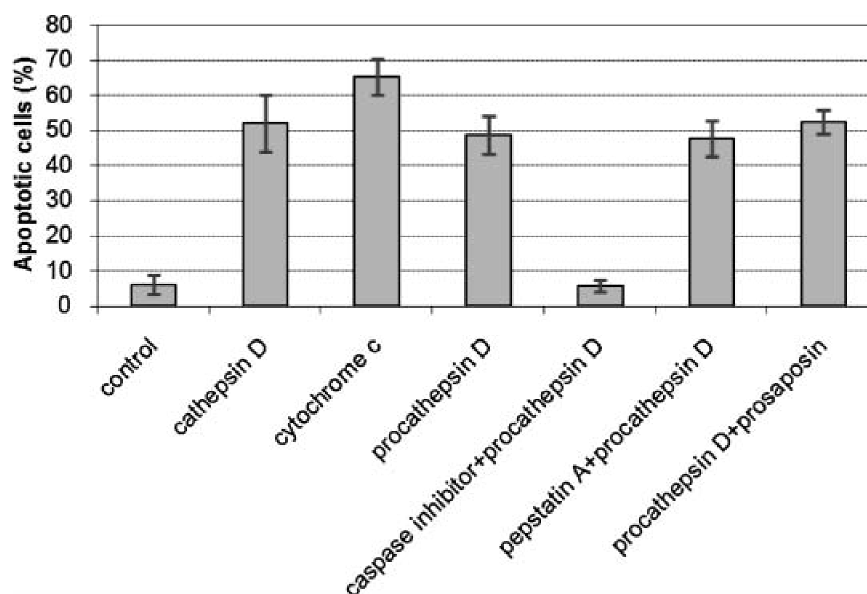

Fig. 5. Statistical evaluation of apoptosis in microinjected HeLa cells after 12 h. The compositions of the microinjected solutions are described in the legend of Figure 3.

and mutated inactive cathepsin D are translocated to the cytosol, and increased the induction of a caspase-dependent apoptosis. In this system pretreatment of cells with pepstatin A did not prevent apoptosis.

Numerous reports implicate cathepsin D in regulation of proliferation, angiogenesis, and apoptosis [Berchem et al., 2002]. Some of these effects seem to depend on its catalytical activity, some on non-catalytic interactions with the apoptotic or the anti-apoptotic machinery. Our report describes systems in which this interaction depends on molecular structures shared by cathepsin D and its precursor. This finding may promote the search for the downstream effectors of lysosomal permeabilization and of the cytosolic cathepsin D.

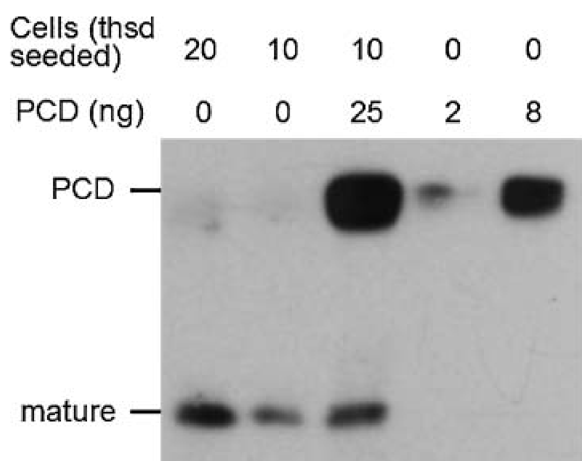

Fig. 6. Western blot analysis of microinjected procathepsin D. Approximately 6,000 cells were microinjected with 1 mg/ml Alexa Fluor in PBS pH 7.0 together with 0.5 mg/ml procathepsin D and incubated for 8 h. Lysates of microinjected and control cells and comparable amounts of procathepsin D dissolved in PBS were subjected to SDS–PAGE and Western blotting. The positions of the precursor (PCD, 53 kDa) and the large mature chain (31 kDa) of cathepsin D are indicated at the margin.

It may be speculated that certain apoptotic signals may be generated under acidic conditions within lysosomes. This proapoptotic function of cathepsin D may be sensitive to pepstatin A. Cytosolic effects of cathepsin D may result in a permeabilization of mitochondria and exit of cytochrome c into cytosol with a subsequent activation of caspases [Johansson et al., 2003]. Such effects may be independent of the enzymatic activity of cathepsin D and explain the apoptotic activity of procathepsin D. The permeabilization of the outer mitochondrial membrane [Garrido et al., 2006] and of lysosomes [Kroemer and Jäättelä, 2005] have been subject to recent reviews. Our results combined with the observation of cytochrome c exit from mitochondria in the presence of cytosolic cathepsin D [Johansson et al., 2003] suggest that the latter promotes a detachment of cytochrome c and/or a formation of pores in the mitochondrial outer membrane. Domains that are not shared by the two forms, the active site cleft and the

prosequence as well as the residues 98-102 processing loop seem to be exempt from the proapoptotic actions of cathepsin D.

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