Mannose 6-Phosphorylated Proteins Are Required for Tumor Necrosis Factor-induced Apoptosis

DEFECTIVE RESPONSE IN I-CELL DISEASE FIBROBLASTS*

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Whereas caspases are essential components in apoptosis, other proteases seem to be involved programmed cell death. This study investigated the role of lysosomal mannose 6-phosphorylated proteins in tumor necrosis factor (TNF)-induced apoptosis. We report that fibroblasts isolated from patients affected with inclusion-cell disease (ICD), having a deficient activity of almost all lysosomal hydrolases, are resistant to the toxic effect of TNF. These mutant cells exhibited a defect in TNF-induced caspase activation, Bid cleavage, and release of cytochrome c. In contrast, TNF-induced p42/p44 MAPK activation and CD54 expression remained unaltered. Human ICD lymphoblasts and fibroblasts derived from mice nullizygous for Ifg2 and the two mannose 6-phosphate (M6P) receptors, Mpr300 and Mpr46, which develop an ICD-like phenotype, were also resistant to CD95 ligand and TNF, respectively. Moreover, correction of the lysosomal enzyme defect of ICD fibroblasts, using a medium enriched in M6P-containing proteins, enabled restoration of sensitivity to TNF. This effect was blocked by exogenous M6P but not by cathepsin B or L inhibitors. Altogether, these findings suggest that some M6P-bearing glycoproteins modulate the susceptibility to TNF-induced apoptosis. As a matter of fact, exogenous tripeptidyl peptide 1, a lysosomal carboxypeptidase, could sensitize ICD fibroblasts to TNF. These observations highlight the hitherto unrecognized role of some mannose 6-phosphorylated proteins such as tripeptidyl peptide 1 in the apoptotic cascade triggered by TNF.

Lysosomes are cytoplasmic organelles that form part of a complex intracellular recycling system involved in the degradation of macromolecules. Interest in lysosomes and lysosomal enzymes is justified by the fact that over 50 lysosomal storage diseases have been described in humans, resulting from the lack or deficient activity of lysosomal hydrolases, transporters, or integral membrane proteins (1–4). Very recently, the concept that alteration of programmed cell death could be related to the development of cell and tissue lesions seen in lysosomal storage diseases, and particularly in neurological diseases, has emerged but still remains to be elucidated (for review see Ref. 5). Indeed, although active lysosomal proteases normally reside in the lysosome and carry out nonspecific degradation of proteins, several lines of evidence indicate that some of them may participate in the control of cell death triggered by cytotoxic drugs (6–10). First, agents that disrupt lysosomes and cause cathepsins to redistribute to the cytoplasm inevitably result in apoptosis induction (11–17). Second, pharmacological (18–23) or endogenous (20, 24, 25) inhibition of cathepsins can block apoptosis. Third, depletion of cathepsin D protein by RNA interference results in the suppression of apoptotic signaling pathways in staurosporine-treated cells (26). Fourth, cells from cathepsin-deficient mice were found to be more resistant to stress-induced apoptosis than their normal counterparts. Indeed, studies on cathepsin D-knockout mice suggest that this aspartyl protease plays a pivotal role in cell death induced by TNF1 (27), doxorubicin, etoposide (28), or α-tocopheryl succinate (29). Similarly, studies on cathepsin B-knockout mice have underscored the role of this cysteine protease in TNF-induced hepatocyte apoptosis and liver injury (30, 31).

However, recent findings have challenged this concept. A series of studies failed to show a requirement for cathepsins or other lysosomal proteases in the cell death machinery but rather suggested a role of these enzymes in cell growth and tissue homeostasis. Indeed, homozygous cathepsin D-deficient mice exhibit increased apoptosis in the thymus (32), thalamus (33), and retina (34). Similar neuronal storage and death have been found in cathepsin D-deficient sheep (35). An increased photoreceptor cell death has also been noticed in a transgenic mouse model expressing an enzymatically inactive cathepsin D (36). Similarly, mice lacking cathepsin B and L display brain atrophy because of massive apoptosis of selected neurons in the cerebral cortex and the cerebellar Purkinje and granular cell layers (37). Elsewhere, another lysosomal protease, palmitoyl-protein thioesterase 1, might also be involved in cell death signaling, because overexpression of palmitoyl-protein thioesterase 1 could protect human neuroblastoma cells against apoptotic death (38). Conversely, its inhibition increases the susceptibility of these cells to apoptosis (39).

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The abbreviations used are: TNF, tumor necrosis factor-α; A-DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; ICD, inclusion-cell disease; MAPK, mitogen-activated protein kinase; M6P, mannose 6-phosphate; MPR, M6P receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly-ADP-ribose polymerase; TFP1, tripeptidyl peptide 1; XIAPs, X-linked inhibitor of apoptosis proteins; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid.
These divergent observations prompted us to address the hitherto unresolved question regarding the involvement of lysosomes in apoptosis. To this end, we took advantage of a genetic model, i.e. cells isolated from patients affected with mucolipidosis II. This disorder, also called inclusion-cell disease (ICD), is characterized by the deficient activity of UDP-N-acetylglucosamine: glycoprotein N-acetylglucosamine-1-phosphotransferase, resulting in the synthesis of lysosomal enzymes that lack mannose 6-phosphate (M6P) residues (40, 41). These lysosomal enzymes fail to bind to the M6P receptors (MPRs) in the trans-Golgi network and are therefore not targeted to the lysosomal compartment but rather quantitatively secreted (42). This results in gross cellular deficiencies of most lysosomal enzymes and is associated with severe clinical manifestations in the first few years of life (43). Clinical, biochemical, and morphological similarities between human ICD, and two conditions in animals have been reported, including in a cat (44) and in triple-deficient mice nullizygous for Igf2, Mpr300 and Mpr46 (45, 46).

The present study evaluated the importance of lysosomal proteases in TNF-induced apoptosis of human or murine ICD cells. We show that TNF-induced cell death was strongly inhibited in ICD fibroblasts as compared with control cells. This phenomenon is likely due to functional inefficiency of some lysosomal hydrolases resulting from their nonlysosomal compartmentalization. Indeed, the apoptosis defect could be partially corrected when the activity of lysosomal hydrolases was restored in ICD fibroblasts, after binding to the MPRs, indicating the importance of these enzymes in cell death. The participation of tripeptidyl peptidase 1, a lysosomal serine-carboxypeptidase, in TNF-induced apoptosis is demonstrated.

Fig. 1. I-cell disease fibroblasts are partially resistant to TNF-induced cytotoxicity. A, SV40-transformed control and I-cell disease (ICD) fibroblasts were incubated for the indicated times in DMEM containing 1% FCS and 50 μg/ml cycloheximide in the presence or absence of TNF (50 ng/ml). Cell viability was assessed using the MTT assay and is expressed as percentage of the viability of cells treated with cycloheximide only. Results are means ± S.E. of 4–11 independent determinations (all in triplicate). B, different SV40-transformed fibroblast cell lines from control subjects and ICD patients were incubated for 24 h in medium containing 1% FCS with 50 ng/ml TNF and 50 μg/ml cycloheximide. Results are means ± S.E. of 3–20 independent determinations. C, SV40-transformed control and ICD fibroblasts were incubated for 24 h in 1% FCS in the presence of 50 μg/ml cycloheximide and the indicated concentrations of TNF. Results are means ± S.E. of three independent experiments. D, untransformed control and ICD3 fibroblasts were incubated for 24 h in 1% FCS in the presence of 50 μg/ml cycloheximide and the indicated concentrations of TNF. Results are means ± S.E. of three independent experiments.

EXPERIMENTAL PROCEDURES

Reagents—Human recombinant TNF was purchased from Peprotech-Tebu (Le-Perray-en-Yvelines, France). Anti-CD95 (clone CH-11) was from Beckman-Coulter (Marseille, France), and CD95 ligand was recovered in the culture medium of transfected Neuro2A cells, which overexpress a murine CD95 ligand (47). Ac-Asp-Glu-Val-Asp-amino-methylcoumarin (Ac-DEVD-AMC) was from Bachem (Voisins-Le-Bretonneux, France). RPMI 1640 Glutamax, DMEM, trypsin-EDTA, fetal calf serum (FCS), penicillin, and streptomycin were from Invitrogen. Other reagents, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and D-mannose 6-phosphate, were supplied from Sigma (Lisle d’Abeau, France).

Cell Culture—Human SV40-transformed skin fibroblasts were derived from normal individuals or from patients affected with ICD (48). Murine fibroblasts derived from MPR46-deficient, MPR300-deficient (49), or triple-deficient mice (null alleles for Igf2, Mpr300, and Mpr46) (45) were kindly provided by Dr. K. von Figura (Gottingen, Germany). These cells were grown in a humidified 5% CO2 atmosphere at 37 °C in DMEM containing Glutamax (2 mM), penicillin, and strepto-
mM phenylmethylsulfonyl fluoride, and 2 anti-ERK2 (Santa Cruz Biotechnology-Tebubio) were used as controls. SV40-transformed control and ICD fibroblasts and disease fibroblasts.

measured with the Bio-Rad dye reagent using bovine serum albumin as a standard.

by Dr. P. Lobel (Piscataway, NJ) and cultured in DMEM. RPMI 1640 medium containing 10% FCS. CHO cells engineered to control subjects or from patients affected with ICD and were grown in G418 (100 μg/ml), and heat-inactivated FCS (10%) (48). Human Epstein-Barr virus-transformed lymphoid cell lines were derived from control subjects or from patients affected with ICD and were grown in RPMI 1640 medium containing 10% FCS. CHO cells engineered to overexpress and secrete TPFP1 (CHO-TPFP1) (50) were kindly provided by Dr. P. Lobel (Piscataway, NJ) and cultured in DMEM.

Cytotoxicity Assay—Cell viability was evaluated by using the tetrazolium-based MTT assay (51).

Fluorogenic DEVD Cleavage Enzyme Assay—After incubation with TNF and cycloheximide, cells were sedimented and washed with phosphate-buffered saline. Cell pellets were homogenized in 10 mM HEPES (pH 7.4), 42 mM KCl, 5 mM MgCl₂, 0.5% CHAPS, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin. Reaction mixtures contained 100 μl of cell lysates and 100 μl of 40 μM Ac-DEVD-AMC. After 30-min incubation at room temperature, the amount of the released fluorescent product aminomethylcoumarin (AMC) was determined at 351 and 430 nm for the excitation and emission wavelengths, respectively.

Release of Cytochrome c—After incubation with TNF and cycloheximide, cells were sedimented and washed with phosphate-buffered saline. Cell pellets were resuspended in 5 volumes of ice-cold homogenization buffer (20 mM HEPES/KOH (pH 7.4), 1 mM EDTA, 0.1% fatty acid-free bovine serum albumin, 250 mM sucrose, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml peptatin A). After swelling for 10 min on ice, cells were homogenized by 15 strokes of a loose-fitting Dounce homogenizer. The suspension was then centrifuged at 750 g for 5 min at 4 °C, and post-nuclear supernatants were centrifuged at 10,000 g for 15 min at 4 °C. Equal amounts of protein were then analyzed by SDS-PAGE (15% gel) and Western blotting by using 1 μg/ml anti-cytocrome c mAb (BD Biosciences).

Western Blot Analyses—Equal amounts of proteins were separated in a 10–15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). Proteins were detected using an ECL detection system (Fierce). Caspase-3 was detected by using a rabbit polyclonal antiserum (BD Biosciences). Goat anti-rabbit and anti-mouse second-antiserum (Cell Signaling, Le-Perray-en-Yvelines, France); p42/p44-phosphorylated MAPK by using a mouse monoclonal antiserum (Cell Signaling); and XIAP by using a mouse monoclonal antiserum (BD Biosciences). Goat anti-rabbit and anti-mouse secondary antibodies were from Cell Signaling. An anti-β-actin (Sigma) or an anti-ERK2 (Santa Cruz Biotechnology-Teubuio) was used as controls for protein loading.

Lysosomal Enzyme Assays—β-Hexosaminidase, β-galactosidase, and β-glucuronidase activities were determined using 0.5 mg/ml of the appropriate 4-methylumbelliferyl derivative substrate (Sigma) at pH 4.5 (β-hexosaminidase) or 3.7 (β-galactosidase and β-glucuronidase), in the presence of 0.1% Triton X-100 (52). Protein concentration was measured with the Bio-Rad dye reagent using bovine serum albumin as a standard.

Cathepsin B activity was assayed with 5 μM Z-Arg-Arg-AMC (Calbiochem) in the presence of 10 μM CA-074Me (Calbiochem), a specific inhibitor of cathepsin B. Cell pellets were homogenized in 250 μl of sodium acetate buffer (0.3 M, pH 5) with 0.1% Triton X-100, 60 mM acetic acid, 4 mM EDTA, and 8 mM dithiothreitol and incubated for 15 min at 4 °C. Reaction mixtures contained 100 μl of cell suspension and 100 μl of substrate preparation. After 30-min incubation at 37 °C, the reaction was stopped by adding 1 ml of 100 mM sodium chloroacetate. The amount of the released fluorescent product AMC was determined by fluorometry.

Cathepsin L activity was assayed with Z-Phe-Arg-AMC 20 μM (Bachem) in the presence of 5 μM CA-074Me. Cell pellets were homogenized in 500 μl of sodium acetate buffer (0.1 M, pH 4.5) with 2 mM EDTA and 10 mM dithiothreitol and incubated for 15 min at 4 °C. Reaction mixtures contained 100 μl of cell suspension and 100 μl of substrate preparation. After 30-min incubation at 37 °C, the reaction was stopped and the amount of AMC was determined as described above for cathepsin B.

Tripeptidyl peptidase 1 (TPFP1) activity was assayed with 100 μM H-Ala-Ala-Phe-AMC (Bachem). Cell pellets were homogenized in 500 μl of sodium acetate buffer (0.1 M, pH 4.5) with 2 mM EDTA and 10 mM dithiothreitol and incubated for 15 min at 4 °C. Reaction mixtures contained 150 μl of supernatant and 50 μl of substrate preparation. After 30-min incubation at 37 °C, the reaction was stopped and the amount of the released fluorescent product AMC was determined by fluorometry.

Flow Cytometry Analyses—TNFR1 expression was analyzed on resting cells. For the analysis of CD54 expression, cells were incubated in medium containing 1% FCS for 24 h in the presence or absence of 50 ng/ml TNF. Then, cells were detached using phosphate-buffered saline containing 10 mM EDTA, sedimented at 4 °C, and washed with phosphate-buffered saline. Cells were incubated at 4 °C in the dark for 30 min with fluorescein isothiocyanate-conjugated mouse anti-human TNFR1 (BD Systems, Lille, France) or with fluorescein isothiocyanate-conjugated mouse anti-human CD54 (Immunotech, Marseille, France). Isotype control monoclonal antibody (Immunotech) was used.
as a negative control. Cytometric analyses were performed on a FACScan (BD Biosciences) cytometer.

Enzymatic Correction Assay—Fibroblasts from wild-type or triple-deficient mice were incubated for 72 h in DMEM containing 1% FCS. Then, the culture medium of these cells was harvested (centrifuged and filtrated) and added to ICD fibroblasts for 48 h. Similar experiments were carried out using the medium of CHO or CHO-TPP1 cells.

Statistical Analyses—Data are presented as means ± S.E. Student’s t test was used for statistical analysis (*, p < 0.05; **, p < 0.01; and ***, p < 0.001).

RESULTS

I-cell Disease Fibroblasts Are Partially Resistant to TNF Toxicity—To investigate the role of lysosomal hydrolases in TNF-induced cell death, we compared the effects of TNF on human SV40-transformed (Fig. 1, A–C) or untransformed (Fig. 1D) fibroblasts derived from normal individuals with those on fibroblasts isolated from ICD patients, in which the activity of almost all lysosomal enzymes is severely impaired (53). The cytotoxic effect of TNF on control cells, in the presence of cycloheximide, was time (Fig. 1A)- and dose (Fig. 1C)-dependent. Under all conditions tested, ICD fibroblasts were more resistant to the lethal effect of TNF than their normal counterparts. This finding was confirmed on several cell lines derived from different normal subjects or patients affected with ICD (Fig. 1B and D), suggesting that some lysosomal enzymes are key effectors of TNF-induced cytotoxicity in human fibroblasts. In addition, ICD fibroblasts were also partially resistant to staurosporine-induced cell death (53).

Because TNFR1 is a well known death receptor able to activate the caspase cascade (54, 55), the implication of lysosomal proteases in the apoptotic cell death induced by TNF in ICD transformed fibroblasts was examined. Executioner caspase activity, as measured by the cleavage of the fluorogenic tetrapeptide substrate DEVD-AMC, increased in normal cells within 2 h, peaking at 4 h post-treatment (Fig. 2). Activation of caspase-3 (i.e. the decrease in the procaspase form and appearance of cleaved forms) was further demonstrated by Western blot analysis (Fig. 3D) and by measuring the cleavage of PARP, a well known substrate of caspase-3 (Fig. 3E). Of interest was the finding that these TNF-induced apoptotic events were impaired in ICD cells. TNF treatment of normal cells also resulted in cleavage of the initiator caspase-8 (Fig. 3A), which occurred quite concomitantly to that of effector caspases (Fig. 3D). The cleavage of caspase 8, as monitored by the disappearance of the proform (not shown) and increase in the cleaved forms, was accompanied by the disappearance of Bid, a member of the Bcl-2 family known as a substrate of caspase-8 (Fig. 3B). Progressive release of cytochrome c was also seen in cytosolic, mitochondria-free extracts from TNF-treated control cells (Fig. 3C). Processing of both caspase-8 and Bid, as well as the release of cytochrome c in the cytosol, were considerably reduced in ICD fibroblasts, further supporting the role of some lysosomal proteins in the apoptotic cascade initiated by TNF with a site of action likely lying upstream of mitochondria. Finally, TNF-induced apoptosis in control fibroblasts was accompanied by a decrease in the content of X-linked inhibitor of apoptosis proteins (XIAPs), which are endogenous inhibitors of the terminal

![Fig. 4](image-url). TNF triggers p42/p44 MAPK activation and CD54 expression equally well in control and I-cell disease fibroblasts. A, TNF-induced activation of p42/p44 MAPK. Cells were incubated in 1% FCS with or without 50 ng/ml TNF and 50 μg/ml cycloheximide for the indicated times, and then harvested. Activated (dually phosphorylated; p-MAPK) or total MAPK were analyzed by Western blot. Results are representative of four independent experiments. B, TNF-induced CD54 cell surface expression by control and ICD fibroblasts. Cells were incubated for 24 h in the presence or absence of 50 ng/ml TNF. CD54 expression was analyzed by flow cytometry. C, TNFR1 expression at the surface of control and ICD fibroblasts, as assessed by flow cytometry. The empty profiles denote labeling with the isotype control.
caspase cascade. This phenomenon was not observed in mutant cells derived from ICD patients (Fig. 3E).

TNF-induced p42/p44 MAPK Activation and TNFR1 or CD54 Expression Are Not Impaired in I-cell Disease Fibroblasts—To rule out the possibility that all TNF-induced signaling pathways were affected in ICD cells in an unspecific manner, other biological activities of this cytokine on fibroblasts, such as p42/p44 MAPK activation and CD54 expression, two well established effects of TNF (56), were assessed. Fig. 4 shows that p42/p44 MAPK activation, observed after 15 min of exposure to TNF, appeared unaffected in ICD cells as compared with normal cells (Fig. 4A). Similarly, the expression of adhesion molecules such as CD54 triggered by TNF on fibroblasts was not abolished in deficient cells (Fig. 4B). Of note, ICD and normal cells expressed similar levels of TNF receptor 1 at the plasma membrane (44% versus 41% in ICD and control cells, respectively) (Fig. 4C). These data indicate that lysosomal enzymes are preferentially implicated in TNF-induced apoptotic pathways but do not interfere with other TNF activities.

I-cell Disease Human Lymphoblasts and Murine Fibroblasts Are Also Resistant to Cell Death—To further evaluate the role of lysosomes in apoptosis, we investigated the lethal effect of CD95, a member of the TNF receptor superfamily (57), in Epstein-Barr virus-transformed lymphoid cells derived from patients affected with ICD. As illustrated in Fig. 5A, the dose-dependent cytotoxic effect of an agonistic anti-CD95 antibody was considerably reduced in ICD lymphoblasts. The resistance of ICD lymphoblasts was further confirmed by examining the cytotoxic effect of recombinant CD95 ligand (Fig. 5A). These data suggest that the resistance of ICD cells to death inducers is not cell-type specific.

We then analyzed the response to TNF of fibroblasts derived from triple-deficient mice nullizygous for Igf2 and the two MPRs, Mpr300, Mpr46, which develop a phenotype that mimics the morphological and biochemical alterations characteristic of ICD (45). As compared with control murine cells, triple-deficient fibroblasts were dose-dependently resistant to TNF (Fig. 5C).

However, the cytotoxic effect of TNF was not altered in cells derived from mice that are deficient for only one of the two MPRs (i.e., either the ~46-kDa cation-dependent MPR or the ~300-kDa cation-independent MPR/insulin-like growth factor-II receptor) (Fig. 5B). This indicates that the resistance of triple-deficient murine cells is not correlated with the absence of one particular MPR, but rather with the deficiency of some mannose 6-phosphorylated proteins.

Correction of the Lysosomal Enzymatic Defect of I-cell Disease Fibroblasts Restores Their Sensitivity to TNF—To determine whether the resistance of ICD fibroblasts to TNF-triggered cell death was due to the absence of some lysosomal hydrolases, we next investigated the effect of lysosomal enzymatic correction of these cells on their sensitivity to TNF.

To this end, human ICD fibroblasts were incubated with the extracellular medium of murine triple-deficient fibroblasts nullizygous for Mpr300, Mpr46, and Igf2. Indeed, deficiency of MPRs in triple-deficient fibroblasts results in abundant secretion of M6P-containing lysosomal enzymes in the culture medium (46). In this medium, the activities of two lysosomal enzymes, β-hexosaminidase and β-galactosidase, were dramatically increased, as compared with the medium of wild-type murine cells (Fig. 6A). Elsewhere, in fibroblasts from ICD patients, deficiency of the phosphotransferase has been shown to result in the synthesis of lysosomal enzymes that lack M6P residues. These lysosomal hydrolases fail to bind to the MPRs and are therefore not routed to lysosomes but secreted (1, 42). When human ICD fibroblasts were incubated for 48 h with the extracellular medium of murine triple-deficient fibroblasts, the intracellular activity of lysosomal enzymes (e.g., β-hexosaminidase and β-glucuronidase) was restored in ICD cells (Fig. 6B). No significant restoration of enzyme activity was achieved when ICD fibroblasts were incubated with the medium of wild-type murine cells.

Of particular interest was the finding that ICD fibroblasts, incubated with the extracellular medium of murine triple-de-
icient fibroblasts, became more sensitive to TNF-induced cytotoxicity, as compared with ICD cells incubated with the medium of control murine cells (Fig. 6C), strongly suggesting that mannose 6-phosphorylated proteins are involved in the apoptotic process triggered by TNF.

To further establish that enzymatic correction of ICD fibroblasts depends on the recognition of M6P-containing proteins by MPRs, we tried to block the uptake of lysosomal proteins by saturating these receptors by excessive amounts of exogenous M6P (58) prior to study TNF-induced cytotoxicity. As shown in Fig. 7A, the cellular uptake of β-hexosaminidase was strongly reduced in the presence of M6P, confirming that the correction of the lysosomal enzymatic defect of ICD fibroblasts required binding of M6P-bearing glycoproteins to their receptors. Under these conditions, i.e. when MPRs were blocked, ICD fibroblasts remained resistant to TNF as compared with cells incubated with the extracellular medium of murine triple-deficient fibroblasts (Fig. 7B).

Effect of Cathepsin Inhibitors on TNF-induced Cell Death in I-cell Disease-corrected fibroblasts, Potential Role of Tripeptidyl Peptidase 1—To assess the possible contribution of cathepsins in the sensitization to TNF of ICD fibroblasts, after enzymatic correction, several protease inhibitors were tested. Previous studies showed that pepstatin A, an aspartic protease inhibitor, did not inhibit TNF-induced cell death (53). As shown in Fig. 8 (A and B), although the purported specific cathepsin B inhibitor CA-074Me strongly inhibited both cathepsin B and L activities, it failed to protect corrected ICD fibroblasts from TNF-induced cell death (Fig. 8C). Similar results were obtained when the extracellular medium of murine triple-deficient fibroblasts was preincubated with (z-Phe-Phe-fmk), a cathepsin L inhibitor. These observations suggest that cathepsins B and/or L, which are only partially deficient in ICD (53), do not participate to TNF signaling of cell death in this system.

We also assessed the possible involvement of another lysosomal protease, tripeptidyl peptidase 1 (TPP1), which is defec-
Activity of this peptidase was increased in ICD cells after incubation with the medium of triple-deficient fibroblasts (data not shown). We tested whether exogenous TPP1 could sensitize ICD fibroblasts to the cytotoxic effect of TNF. Incubation of ICD cells with the extracellular medium of CHO cells overexpressing and secreting TPP1 led to an increase in the intracellular activity of TPP1 and sensitivity to TNF, suggesting that TPP1 may contribute to TNF-induced cell death.

**DISCUSSION**

For many years, lysosomes have been viewed solely as a reservoir of nonspecific hydrolases in the mammalian cell. This concept has recently been revisited, pointing to the potential role of these organelles to programmed cell death. However, the involvement of lysosomes in apoptosis is still debated. Not only because the precise mechanisms by which lysosomes are involved in apoptosis are still unknown, but also because no clear correlation between the phenotype observed in mice lacking one particular lysosomal enzyme and a disturbed apoptosis in tissues of the affected animals has been established.

The present work aimed at testing the hypothesis that lysosomal proteases play an active role in the execution phase of programmed cell death.

**Place of Lysosomes in TNF-induced Apoptosis**—Based on the use of a genetic model, i.e. cells characterized by a mistargeting of lysosomal enzymes, the present findings demonstrate that the apoptotic effect of TNF or CD95 ligand involves a signal emanating from lysosomal components. In ICD cells, TNF-induced cytochrome c release into the cytosol was impaired, as was the cleavage of effector caspase-3 and its substrate PARP. Inhibition of XIAP, due to its interaction with Smac/Diablo, was also strongly attenuated in mutant cells. In addition, cleavage of Bid, a pro-apoptotic member of the Bcl-2 family, which controls the mitochondrial checkpoint of apoptosis in different models, was inhibited in ICD cells. These data might indicate that Bid is essential for lysosome-directed apoptosis in agreement with several reports. However, as the defect in lysosomal enzymes markedly reduced activation of initiator caspase-8 and because a comparable time course of activation was observed for caspase-8 and caspase-3, the possibility that caspase-8 is (also) activated downstream of mitochondrial events cannot be ruled out. This would be consistent with the observation that caspase-8 activation induced by staurosporine, a typical receptor-independent inducer of apoptosis, was also inhibited in ICD cells.

The nature of the one or more factors that mediate resistance of ICD cells to death receptor-induced apoptosis was further investigated in ICD fibroblasts (Fig. 9A). Activity of this peptidase was increased in ICD cells after incubation with the medium of triple-deficient fibroblasts (data not shown). We tested whether exogenous TPP1 could sensitize ICD fibroblasts to the cytotoxic effect of TNF. Incubation of ICD cells with the extracellular medium of CHO cells overexpressing and secreting TPP1 led to an increase in the intracellular activity of TPP1 and sensitivity to TNF, suggesting that TPP1 may contribute to TNF-induced cell death.

**FIG. 7.** Mannose 6-phosphate blocks the uptake of lysosomal enzymes in human I-cell disease fibroblasts and leads to resistance of these cells. ICD fibroblasts were incubated for 48 h with DMEM containing 1% FCS or with the extracellular medium of triple-deficient (3KO) fibroblasts in the presence or absence of M6P (2 mM). Then, β-hexosaminidase activity was determined (A) and TNF-induced cell death was monitored (B). Cells were stimulated with 50 µg/ml cycloheximide in the presence or absence of 50ng/ml TNF for 16 h. Cell viability was assessed by MTT assay. Results are means ± S.E. of 4–12 independent determinations (all in triplicate).

**FIG. 8.** Cathepsins B and L are not implicated in the restoration of TNF-induced cell death in human I-cell disease fibroblasts. ICD fibroblasts were incubated for 48 h with DMEM containing 1% FCS or with the extracellular medium of triple-deficient (3KO) fibroblasts in the presence or absence of the cathepsin inhibitors CA-074Me and z-Phe-Phe-fmk (5 µM). Then, the activity of cathepsin B (A) and cathepsin L (B) was determined, and TNF-induced cell death was monitored (C). Cells were stimulated with 50 µg/ml cycloheximide in the presence or absence of 50 ng/ml TNF for 16 h. Cell viability was assessed by MTT assay. Results are means ± S.E. of 3 independent determinations (all in triplicate).
investigated using MPR-deficient cell lines. First, the observation, that fibroblasts lacking either MPR46 or MPR300, which secrete large quantities of lysosomal enzymes (49) as ICD cells do, were as sensitive as their parental counterparts, suggests that the resistance of ICD cells is not linked to an extracellular action of lysosomal hydrolases (e.g. on membrane receptors). This is also supported by the fact that the medium of triple-deficient cells that is enriched in lysosomal enzymes did not confer sensitivity to normal cells. Second, resistance of ICD cells to TNF is not due to insulin-like growth factor II or another nonmannose 6-phosphorylated ligand, because MPR300-deficient cells were fully sensitive. Finally, enzymatic correction and sensitization of ICD fibroblasts to TNF-induced cell death was monitored (A), and TNF-induced cell death was monitored (B). Cells were stimulated with 50 μg/ml cycloheximide in the presence or absence of 50 ng/ml TNF for 16 h. Cell viability was assessed by MTT assay. Results are means ± S.E. of four independent determinations (all in triplicate).

Three lysosomal proteases, cathepsins B, D, and L, are potential candidates to play a critical role in apoptosis (10), but how they promote cell death after being released into the cytosol is not clearly defined. Indeed, direct activation of caspases by cathepsins remains to be proven, because effector caspases, such as procaspase-3 and -7, have been shown to be poor substrates for various cathepsins in vitro (60, 66). The effect of cathepsins on mitochondria via the cleavage of Bid is also controversial, because Bid has been reported to be cleaved to the same extent in wild-type and cathepsin B-deficient hepatocytes in vitro and in vivo after TNF treatment (10). Because lysosomal extracts have been shown to cleave Bid in vitro to generate a fragment with cytochrome c-releasing activity (60), this may indicate that lysosomal proteases other than cathepsin B are implicated in the apoptotic pathway. This is further substantiated by the fact that pharmacological inhibition of cathepsin B, L, and D activities does not suppress Bid cleavage or caspase activation induced by photodynamic therapy in murine hepatoma cells (67).

Consistent with these observations, the present study shows that cathepsin B and L activities do not correlate with the sensitization of ICD fibroblasts to TNF-induced apoptosis. Interestingly, restoration of TPP1 activity resulted in an increased susceptibility of ICD cells to TNF cytotoxicity, suggesting the potential role of this lysosomal peptidase in cell death. This is, to our knowledge, the first report documenting a possible implication of TPP1 in TNF-induced apoptosis. Human TPP1 is a lysosomal serine protease with an acidic pH optimum that cleaves off tripeptides from the free N termini of small polypeptides (68, 69). However, its natural substrates are unknown. The activity of the mature enzyme is not inhibited by standard serine, cysteine, metallo, or aspartyl protease inhibitors (70). Naturally occurring mutations in TPP1 are associated with a fatal lysosomal disorder, the classic late infantile form of neuronal ceroid lipofuscinosis (CLN2 or Jansky-Bielschowsky disease) (71, 72), which is accompanied by a disturbed apoptosis (73). Of note, another tripeptidyl peptidase, TPP2, a cytoplasmic enzyme that belongs to the subtilisin class of serine proteases (74), has been implicated in apoptosis induced by the bacterium *Shigella flexneri* and staurosporine in macrophages (75). Whether TPP1 is released in the cytosol and how this protease is connected to the cell death machinery needs to be clarified.

**Ceramide and Lysosomes in TNF Signaling**—The sphingomyelin-ceramide pathway has received a lot of attention in part due to its role as a key element involved in apoptosis. Indeed, although important issues regarding the mechanisms of cer-

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3 The incomplete resensitization of ICD cells to TNF-induced apoptosis by TPP1 might suggest that additional lysosomal peptidases or proteinases are required for TNF cytotoxicity. Preliminary observations from our laboratory on different mutant cell lines indicate that other enzymes than TPP1 are indeed involved.
amide generation and mode of action still remain to be addressed, previous studies identified ceramide as a potential second messenger in the apoptotic cascade triggered by TNF (76). Ceramide generation was shown to result from the stimulation of a sphingomyelinase activity and the concomitant hydrolysis of the membrane lipid sphingomyelin. A connection between these lipid messengers and the lysosomal pathway of cell death has been recently proposed. Indeed, the endolysosomal aparycell caspase cathepsin D has been reported to be regulated by sphingosine (77) and ceramide derived from the hydrolysis of sphingomyelin by acid sphingomyelinase (27, 78). The interaction between ceramide and cathepsin D has been proposed to result in autocalytic proteolysis of the pre-pro-cathepsin D to form the enzymatically active isoforms of the protease (78). In contrast to these observations, a recent report demonstrates that the transport and processing of cathepsin D are inhibited during ceramide-induced apoptosis in human colon adenocarcinoma cells (79).

Moreover, the binding of the adapter protein FAN (Factor Associated with Neutral sphingomyelinase activation) to the TNFR1 receptor has been associated with ceramide generation and apoptosis (80) as well as lysosomal permeabilization with release of cathepsin B into the cytosol (17). Altogether, these findings suggest that stress-induced ceramide generation precedes activation of the lysosomal pathway, in agreement with our previous study (53). However, further investigations are needed to clarify how, where, and with which proteases the ceramide pathway is connected with the lysosome in TNF-induced apoptosis.

In summary, the present study demonstrates that a normal lysosomal function implicating mannose 6-phosphorylated enzymes is required for efficient cell death. Our finding that TPP1 can play an active role in TNF-induced cell death may provide critical information for understanding the pathophysiology of lysosomal storage diseases and more particularly for the late infantile form of neuronal ceroid lipofuscinosis.
Role of Mannose 6-Phosphorylated Proteins in Apoptosis

52923

Vos, K., Totty, N., Sterling, A., Fiers, W., Vandenabeele, P., and Grooten, J. (1998) FEBS Lett. 438, 150–158
67. Reiners, J. J., Jr., Caruso, J. A., Mathieu, P., Chelladurai, B., Yin, X. M., and Kessel, D. (2002) Cell Death Differ. 9, 934–944
68. Lin, L., Sohar, I., Lackland, H., and Lobel, P. (2001) J. Biol. Chem. 276, 2249–2255
69. Golabek, A. A., Kida, E., Walus, M., Wujek, P., Mehta, P., and Wisniewski, K. E. (2003) J. Biol. Chem. 278, 7133–7145
70. Vines, D., and Warburton, M. J. (1998) Biochem. Biophys. Acts 1384, 233–242
71. Sleat, D. E., Donnelly, R. J., Lackland, H., Liu, C. G., Sohar, I., Pullarkat, R. K., and Lobel, P. (1997) Science 277, 1802–1805
72. Vines, D. J., and Warburton, M. J. (1999) FEBS Lett. 443, 131–135
73. Lane, S. C., Jolly, R. D., Schmechel, D. E., Alroy, J., and Boustany, R. M. (1996) J. Neurochem. 67, 677–683
74. Tomkinson, B., Werstfeldt, C., Hellman, U., and Zetterqvist, O. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7508–7512
75. Hilbi, H., Purr, R. J., and Zychlinsky, A. (2000) Infect. Immun. 68, 5502–5508
76. Malagarie-Cazenave, S., Andreieu-Abadie, N., Segui, B., Gouazet, V., Tardy, C., Cuvillier, O., and Levede, T. (2002) Expert Rev. Mol. Med. 2002, 1–15
77. Kagedal, K., Zhao, M., Svensson, I., and Brunk, U. T. (2001) Biochem. J. 359, 335–343
78. Heinrich, M., Wickel, M., Schneider-Brachert, W., Sandberg, C., Gahr, J., Schwandner, R., Weber, T., Saftig, P., Peters, C., Brunner, J., Kronke, M., and Schutze, S. (1999) EMBO J. 18, 5252–5263
79. De Stefani, D., Reffo, P., Benelli, G., Bacino, F. M., Sala, G., Gladoni, R., Codogno, P., and Isidoro, C. (2002) Biol. Chem. 383, 989–999
80. Segui, B., Cuvillier, O., Adam-Klages, S., Garcia, V., Malagarie-Cazenave, S., Levêque, S., Caspar-Bauguil, S., Coudert, J., Salvayre, R., Kronke, M., and Levede, T. (2001) J. Clin. Invest. 108, 143–151