Introduction
Apoptosis is a common, conserved, endogenous cell suicide program required for proper embryonic development and maintenance of homeostasis in adult tissues (Vaux and Korsmeyer, 1999; Jacobson et al., 1997). Apoptosis is characterized by a biochemical cascade and morphological changes, including activation of caspases, translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane, blebbing of cytoplasmic membranes, chromatin condensation and fragmentation into apoptotic bodies (Kerr et al., 1972; Thornberry and Lazebnik, 1998; Hengartner, 2000). Recently, a novel non-apoptotic programmed cell death (PCD) process designated ‘paraptosis’, was described. Here, we show that overexpression of TAJ/TROY, a member of the tumor necrosis factor receptor superfamily, induces non-apoptotic cell death with paraptosis-like morphology in 293T cells. Transmission electron microscopy studies reveal extensive cytoplasmic vacuolation and mitochondrial swelling in some dying cells and no condensation or fragmentation of the nuclei. Characteristically, cell death triggered by TAJ/TROY was accompanied by phosphatidyserine externalization, loss of the mitochondrial transmembrane potential and independent of caspase activation. In addition, TAJ/TROY suppressed clonogenic growth of HEK293 and HeLa cells. Interestingly, overexpression of Programmed cell death 5 (PDCD5), an apoptosis-promoting protein, enhanced TAJ/TROY-induced paraptotic cell death. Moreover, cellular endogenous PDCD5 protein was significantly upregulated in response to TAJ/TROY overexpression. These results provide novel evidence that TAJ/TROY activates a death pathway distinct from apoptosis and that PDCD5 is an important regulator in both apoptotic and non-apoptotic PCD.

Summary
Accumulating reports demonstrate that apoptosis does not explain all the forms of programmed cell death (PCD), particularly in individual development and neurodegenerative disease. Recently, a novel type of PCD, designated ‘paraptosis’, was described. Here, we show that overexpression of TAJ/TROY, a member of the tumor necrosis factor receptor superfamily, induces non-apoptotic cell death with paraptosis-like morphology in 293T cells. Transmission electron microscopy studies reveal extensive cytoplasmic vacuolation and mitochondrial swelling in some dying cells and no condensation or fragmentation of the nuclei. Characteristically, cell death triggered by TAJ/TROY was accompanied by phosphatidyserine externalization, loss of the mitochondrial transmembrane potential and independent of caspase activation. In addition, TAJ/TROY suppressed clonogenic growth of HEK293 and HeLa cells. Interestingly, overexpression of Programmed cell death 5 (PDCD5), an apoptosis-promoting protein, enhanced TAJ/TROY-induced paraptotic cell death. Moreover, cellular endogenous PDCD5 protein was significantly upregulated in response to TAJ/TROY overexpression. These results provide novel evidence that TAJ/TROY activates a death pathway distinct from apoptosis and that PDCD5 is an important regulator in both apoptotic and non-apoptotic PCD.

Key words: Paraptosis, Non-apoptotic, PDCD5, TFAR19, TAJ/TROY

An alternative form of paraptosis-like cell death, triggered by TAJ/TROY and enhanced by PDCD5 overexpression

Ying Wang1, Xianting Li1, Lu Wang2, Peiguo Ding1, Yingmei Zhang2, Wenling Han2 and Dalong Ma1,2,*
1Laboratory of Medical Immunology, School of Basic Medical Science, Peking University, Xueyuan Road 38, Beijing 100083, China
2Center for Human Disease Genomics, Health Science Center, Peking University, Beijing 100083, China
*Author for correspondence (e-mail: madl@bjmu.edu.cn)

Accepted 18 November 2003
Journal of Cell Science 117, 1525-1532 Published by The Company of Biologists 2004
doi:10.1242/jcs.00994
apoptotic PCD (Baker and Reddy, 1998; Gravstein and Borst, 1998). However, little is known about whether members of TNFR are involved in non-apoptotic or paraptotic PCD. TAJ/TROY (HGNC-approved symbol TNFRSF19) is a novel member of the TNFR family that is highly expressed during embryonic development and lacks a death domain in the cytoplasmic tail of its protein product (Eby et al., 2000; Hu et al., 1999; Kojima et al., 2000). Similar to other members of the TNFR family, TAJ/TROY activates JNK (Eby et al., 2000) and NF-kB (Eby et al., 2000; Kojima et al., 2000) pathways. Co-immunoprecipitation assay revealed that TAJ/TROY binds TRAF1, TRAF2, TRAF3 and TRAF5 (Eby et al., 2000). TAJ/TROY is capable of inducing apoptosis independent of DNA fragmentation and caspase activation (Eby et al., 2000), and might transduce signals associated with epidermal development through TRAF6 (Naito et al., 2002; Sinha et al., 2002).

**PDCD5 (programmed cell death 5), formerly designated TFAR19 (TF-1-cell apoptosis-related gene 19), is an apoptosis-promoting gene cloned in our laboratory (Liu et al., 1999). An mRNA dot blot disclosed that PDCD5 is expressed ubiquitously in adult tissues and, at lower levels, in fetal tissues (Liu et al., 1999). PDCD5 mRNA and protein levels are upregulated in response to various apoptotic stimuli (Li et al., 2000; Liu et al., 1999). Overexpression of this gene induces increased sensitivity of specific tumor cells to apoptotic stimulation (Liu et al., 1999). These results suggest that PDCD5 is a positive regulator in classic apoptotic PCD. However, whether PDCD5 is involved in non-apoptotic or paraptotic PCD has never been investigated.

In this report, we provide novel evidence that TAJ/TROY induces non-apoptotic cell death with paraptosis-like morphology in HEK293, HeLa and 293T cells. Significantly, TAJ/TROY-induced paraptotic cell death was enhanced efficiently upon overexpression of PDCD5, which plays a pro-apoptotic role in classical apoptosis. In addition, significant translational upregulation of PDCD5 was observed in TAJ/TROY-transfected cells. These results highlight a signal transduction system to study alternative PCD and suggest that PDCD5 is an important regulator implicated in both apoptotic and paraptotic PCD.

**Materials and Methods**

**Cell lines and reagents**

HEK293, HeLa and 293T (a kind gift from T. Matsuda, Japan) cells were maintained in Dulbecco's modified Eagle medium (Life Technologies, USA) supplemented with penicillin (100 units ml⁻¹), streptomycin (100 μg ml⁻¹), L-glutamine (2 mM) and 10% fetal bovine serum (Hyclone, USA). All experiments were performed on logarithmically growing cells. z-VAD-fmk and Ac-DEVD-AMC were purchased from Enzyme Systems (Hayward, CA, USA). Monoclonal antibodies against caspase-3 (catalog number 610322), XIAP (catalog number 610763) and PARP (catalog number P76420) were obtained from Transduction Laboratories (Becton Dickinson, USA). Monoclonal antibody against b-actin was obtained from Sigma. Rainbow protein marker was obtained from Amersham Pharmacia. Anti-PDCD5 (clone 3A3) monoclonal antibody and labeled FITC-3A3 were prepared in our laboratory (Chen et al., 2001).

**Constructs and transient transfection**

Human TAJ/TROY cDNA was amplified with 10 pmol of following primer 5'-AAGATGGCTTTAAAAGTGTCTAG-3' and 5'-AGTC-TCGAGTCACAGGGAACCCAGTGC-3' using a PCR reaction with 1 cycle of 94°C for 5 minutes and 35 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1.5 minutes, followed by an extension at 72°C for 7 minutes from a fetal heart cDNA library (Clontech, USA). The PCR product was subcloned into pcDNA3.1-Myc-His (B) vector (Invitrogen, USA). The cDNA encoding human Bax was generated by PCR and subcloned into pcDNA3.1-Myc-His (B). The entire open reading frame of human PDCD5 was subcloned from pCDI-PDCD5 (Liu et al., 1999) and inserted into pcDNA3.1-Myc-His (B) with a C-terminal Myc tag using an EcoRI site. All plasmids were confirmed by DNA sequencing. HeLa, HEK293 and 293T cells were transiently transfected using the calcium phosphate precipitation method according to standard protocols (Xia et al., 2002).

**Clonogenic assay**

Briefly, the constructs were transfected into HEK293 and HeLa cells using the calcium phosphate precipitation method. After incubation for 48 hours at 37°C, transfected cells were trypsinized and seeded into 100-mm dishes (1000 cells per dish). Selection medium with G418 (600–800 μg ml⁻¹) was added; 3 weeks later, colonies were fixed and stained with crystal violet, and clones containing more than 50 cells were counted. Each group was assayed in triplicate dishes, and each experiment was repeated twice.

**Electron microscopy**

For transmission electron microscopy, cells were initially fixed in 0.1 M sodium phosphate buffer containing 2.5% glutaraldehyde, pH 7.4. Next, cells were fixed in 0.1 M sodium phosphate buffer containing 1% OsO₄, pH 7.2. Cells were embedded into Ultracut (Leica, Germany) and sliced into 60 nm sections. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEM-1230 transmission electron microscope (JEOL, Japan).

**Detection of phosphatidylserine externalization**

To detect phosphatidylserine (PS) externalization, transfected 293T cells (5×10⁵) were harvested by trypsinization and washed twice with PBS. Washed cells were resuspended in 200 μl binding buffer (PBS containing 1 mM calcium chloride). FITC-conjugated annexin V (0.5 μg ml⁻¹ final concentration) and propidium iodide (PI; 1 μg ml⁻¹ final concentration) were added according to the manufacturer’s instructions (Biosea, China). After incubation for 20 minutes at room temperature, 400 μl binding buffer was added, and samples were immediately analysed on a FACS Calibur flow cytometer (Becton Dickinson, USA) with excitation using a 488 nm argon ion laser. PI was added to samples to distinguish necrotic and late apoptotic events (annexin V–, PI+; annexin V+, PI+) from early apoptotic events (annexin V+, PI–).

**Measurement of mitochondrial membrane potential**

Rhodamine 123 was used to evaluate changes in mitochondrial membrane potential (ΔΨm). Cell suspensions (5×10⁵) were incubated for 15 minutes at 37°C in 1 ml PBS containing 1 mM rhodamine 123 and subsequently analysed with a FACS Calibur flow cytometer. Results were expressed as the proportion of cells exhibiting low mitochondrial membrane potential estimated by the reduced rhodamine 123 uptake.

**DEVDAse activity analysis**

293T cells (3×10⁵) were transfected with 2 μg empty vector and TAJ/TROY or Bax expression vectors. Cells were subsequently washed twice with pre-cold PBS and harvested in lysis buffer (10 mM Tris HCl, pH 7.5, 10 mM Na₂HPO₄/NaH₂PO₄, 130 mM NaCl, 1% OsO₄, pH 7.2. Cells were embedded into Ultracut (Leica, Germany) and sliced into 60 nm sections. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEM-1230 transmission electron microscope (JEOL, Japan).

**Clonogenic assay**

Briefly, the constructs were transfected into HEK293 and HeLa cells using the calcium phosphate precipitation method. After incubation for 48 hours at 37°C, transfected cells were trypsinized and seeded into 100-mm dishes (1000 cells per dish). Selection medium with G418 (600–800 μg ml⁻¹) was added; 3 weeks later, colonies were fixed and stained with crystal violet, and clones containing more than 50 cells were counted. Each group was assayed in triplicate dishes, and each experiment was repeated twice.

**Electron microscopy**

For transmission electron microscopy, cells were initially fixed in 0.1 M sodium phosphate buffer containing 2.5% glutaraldehyde, pH 7.4. Next, cells were fixed in 0.1 M sodium phosphate buffer containing 1% OsO₄, pH 7.2. Cells were embedded into Ultracut (Leica, Germany) and sliced into 60 nm sections. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEM-1230 transmission electron microscope (JEOL, Japan).

**Detection of phosphatidylserine externalization**

To detect phosphatidylserine (PS) externalization, transfected 293T cells (5×10⁵) were harvested by trypsinization and washed twice with PBS. Washed cells were resuspended in 200 μl binding buffer (PBS containing 1 mM calcium chloride). FITC-conjugated annexin V (0.5 μg ml⁻¹ final concentration) and propidium iodide (PI; 1 μg ml⁻¹ final concentration) were added according to the manufacturer’s instructions (Biosea, China). After incubation for 20 minutes at room temperature, 400 μl binding buffer was added, and samples were immediately analysed on a FACS Calibur flow cytometer (Becton Dickinson, USA) with excitation using a 488 nm argon ion laser. PI was added to samples to distinguish necrotic and late apoptotic events (annexin V–, PI+; annexin V+, PI+) from early apoptotic events (annexin V+, PI–).

**Measurement of mitochondrial membrane potential**

Rhodamine 123 was used to evaluate changes in mitochondrial membrane potential (ΔΨm). Cell suspensions (5×10⁵) were incubated for 15 minutes at 37°C in 1 ml PBS containing 1 mM rhodamine 123 and subsequently analysed with a FACS Calibur flow cytometer. Results were expressed as the proportion of cells exhibiting low mitochondrial membrane potential estimated by the reduced rhodamine 123 uptake.

**DEVDAse activity analysis**

293T cells (3×10⁵) were transfected with 2 μg empty vector and TAJ/TROY or Bax expression vectors. Cells were subsequently washed twice with pre-cold PBS and harvested in lysis buffer (10 mM Tris HCl, pH 7.5, 10 mM Na₂HPO₄/NaH₂PO₄, 130 mM NaCl.
1% Triton-X100, 1 mM PMSF). Cell lysates were clarified by centrifugation at 18,000 g for 20 minutes at 4°C. Cell lysates containing 15 µg protein were incubated at 37°C in buffer containing 25 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.1% CHAPS and 10 mM dithiothreitol with the fluorogenic substrate Ac-DEVD-AMC. To measure this signal, we used a FLUOStar fluorometer (BMG Labtechnologies, Germany) with an excitation filter of 380 nm and emission filter of 460 nm. Results were calculated as a proportion of the control over 90 minutes (T90 to T0). Samples were prepared in triplicate.

Protein extraction and immunoblotting
Cells were pelleted by centrifugation, lysis in lysis buffer (20 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF) and incubated for 30 minutes on ice. Lysates were centrifuged at 18,000 g for 10 minutes at 4°C and the supernatant was measured using the BCA protein assay reagent (Pierce, USA). Equal amounts of protein (50 µg) were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Hybond™ ECL™; Amersham Pharmacia, UK). Membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) containing 5% non-fat milk for 1 hour and incubated overnight at 4°C with the appropriate primary antibody. Blots were washed (three times for 10 minutes each) with TBS-T and incubated for 1 hour with the appropriate horseradish-peroxidase-conjugated secondary antibody (Promega, USA) prepared in TBS-T/5% non-fat milk. Blots were washed (three times for 10 minutes each) before detection of immunocomplexes using the enhanced chemiluminescence system (Amersham Pharmacia, UK).

Immunofluorescence analysis
Transfected cells were washed in PBS, fixed in freshly prepared 2% paraformaldehyde (containing 0.1% Triton X-100) for 30 minutes at room temperature, pre-incubated in 2% fetal bovine serum for 1 hour and incubated with FITC-3A3 for 1 hour. Following antibody staining, cells were analysed using a FACSCalibur flow cytometer.

Results
TAJ/TROY as a paraptotic cell death inducer in cultured cells
TAJ/TROY-transfected cells underwent marked rounding and eventually detached from the culture dish. There were no signs of classic apoptotic changes, such as cell shrinkage or membrane blebbing, as observed in Bax-transfected cells. Ultrastructural analyses revealed that TAJ/TROY-induced cell death was distinct from apoptosis. Transmission electron microscopy of HEK293, HeLa and 293T cells at 40 hours after transfection with TAJ/TROY confirmed that these cells did not undergo apoptosis, unlike Bax-transfected cells, which displayed features typical of apoptosis, including chromatin condensation with margination at the nuclear periphery, nuclear fragmentation and apoptotic body formation (Fig. 1B). Compared with untreated cells, significant swelling of mitochondria and ER were observed in TAJ/TROY-transfected cells and the nuclei were intact. Moreover, chromatin condensation and nuclear fragmentation were absent and some cells displayed extensive cytosolic vacuolization. Clonogenic survival assays were performed to determine the death-inducing effect of TAJ/TROY on HEK293 and HeLa cells over an extended period of growth. As shown in Fig. 1A, a significant decrease in the number of colonies was observed for TAJ/TROY-transfected cells compared to mock-transfected cells (B).

![Fig. 1. Overexpression of TAJ/TROY induces non-apoptotic cell death. (A) Colony formation after TAJ/TROY transfection. HEK293 and HeLa cells were transfected with an empty vector or expression vectors encoding Bax or TAJ/TROY. Overexpression of TAJ/TROY significantly decreased clonogenic survival of the cells. The figure shows a representative experiment with the means and standard deviations from triplicate plates. (B) Electron micrographs of TAJ/TROY-induced cell death. HEK293, HeLa and 293T cells were transfected with a vector control or plasmids encoding Bax or TAJ/TROY along with a GFP-encoding vector. Whereas Bax-transfected 293T cells (b) displayed classic characteristics of apoptosis, including chromatin condensation and apoptosis body, TAJ/TROY-transfected HEK293 (c), HeLa (g) and 293T (c) cells showed extensive cytoplasmic vacuolization and swelling of mitochondria or ER, along with absence of nuclear fragmentation, membrane blebbing or apoptotic-body formation. The following micrographs of empty-vector-transfected HEK293 (d) and HeLa (f) and 293T (a) cells provided normal cell controls. Higher-magnification images of the vacuolization suggested that some vacuoles might be formed from the remnants of the mitochondrial (h) or ER (i). The transfection efficiency of the indicated plasmids ranged from 60% to 70% as determined by the expression of co-transfected GFP. Scale bars, 2 µM (a-g), 0.2 µM (h,i).]
survival colonies was observed in the TAJ/TROY transfected cells compared with control cells. Taken together, we propose that TAJ/TROY-transfected cells died through a paraptotic mechanism.

Cell death triggered by TAJ/TROY is accompanied by exposure of PS

A biochemical hallmark of apoptotic cell death is the translocation of PS from the cytoplasmic surface of the cell membrane to the external cell surface (Fadok et al., 1998). Exposure of PS at the surface of apoptotic cells is easily determined by flow cytometry using fluorescence-labeled annexin V, which specifically binds PS (Smith et al., 2002). To characterize TAJ/TROY-induced cell death further, we detected PS surface exposure by FITC/annexin-V staining of cells 40 hours after transfection. Plasma membrane integrity was simultaneously assessed by PI dye exclusion using two-color fluorescence-activated cell sorting (FACS) analysis. In TAJ/TROY-transfected cells, the proportion of annexin-V-positive/PI-negative cells was evidently increased (Fig. 2). Simultaneously, the integrity of the membrane in these cells was maintained, based on the finding that the PI-positive population was similar to that observed in mock-transfected cells.

TAJ/TROY-induced cell death is caspase-independent

Activation of effector caspases, such as caspases 3 and 7, is responsible for the proteolytic cleavage of a diverse range of structural and regulatory proteins in apoptosis (Salvesen and Dixit, 1997). To determine whether PS externalization is related to caspase-3-mediated proteolysis, we analysed the activation of caspase-3 in TAJ/TROY- and Bax-transfected cells approximately 40 hours after transfection. DEVD cleavage assay is a quantitative method used to detect caspase-3-like activity (Rehm et al., 2002). As shown in Fig. 3A, DEVD cleavage activity in lysates of TAJ/TROY-overexpressing cells was at background levels, whereas activity in lysates from Bax-overexpressing cells was nearly ten times higher than that in mock cell lysates. The significant caspase-3-like activity in Bax-transfected cells indicated priming of the caspase cascade for activation. The pan-caspase inhibitor, z-VAD-fmk, effectively inhibited this caspase-3-like activity. To confirm the activation of caspase-3, western blots were performed. We detected decreased procaspase-3 in lysates of Bax-overexpressing cells but not in those of TAJ/TROY-overexpressing or mock cells (Fig. 3B). Because proteolytic cleavage of specific substrates by activated caspases is responsible for cellular dysfunction and structural destruction apoptosis (Thornberry and Lazebnik, 1998), we analysed the cleavage of PARP and XIAP as representative substrates in mock, TAJ/TROY- or Bax-transfected cells. Both substrates were cleaved in Bax-transfected cells and that PARP cleavage led to approximate 80 kDa fragments that is a feature of classic apoptosis (Fig. 3B). No significant caspase-specific processing of PARP and XIAP was observed in TAJ/TROY-transfected cells, strongly suggesting that the caspase cascade is not activated during TAJ/TROY-induced cell death.

Mitochondrial changes in TAJ/TROY-transfected cells

An increasing amount of evidence shows that mitochondria play central roles in the regulation of both apoptotic and non-apoptotic cell death (Green and Reed, 1998; Sperandio et al., 2000; Obrero et al., 2002). Among the sequence of events taking place in mitochondria during the course of cell death, loss of the $\Delta \Psi_m$ appear to be the major event closely associated with cell death (Susin et al., 1996). Accordingly, at 30 hours after transfection, we examined the involvement of mitochondria in TAJ/TROY-induced non-apoptotic cell death by monitoring $\Delta \Psi_m$. During Bax-induced apoptosis, we detected loss of $\Delta \Psi_m$ (Fig. 4A). Interestingly, $\Delta \Psi_m$ loss was additionally observed in TAJ/TROY-induced cell death (Fig. 4A), suggesting that mitochondria are involved in the regulation of non-apoptotic cell death. Ultrastructural analysis at 40 hours after transfection revealed significant mitochondrial swelling in TAJ/TROY-transfected cells and ER swelling also was observed (Fig. 4B). These results suggested that some vacuoles might be derived from the mitochondria or ER.

Overexpression of PDCD5 enhances TAJ/TROY-induced cell death

PDCD5 is a pro-apoptotic regulator. We examined whether the protein has synergistic effects on TAJ/TROY-mediated paraptotic cell death. 36 hours after transfection, cells were photographed under a light microscope. Co-transfection of TAJ/TROY and PDCD5 resulted in a more severe cytotoxic effect on 293T cells than TAJ transfection alone (data not shown). Annexin-V staining revealed more positive cells in the co-transfection group than the TAJ/TROY transfection group. However, there were no differences in the number of annexin-V-positive cells between mock-transfected and PDCD5-transfected cells (Fig. 5A). Therefore, PDCD5 clearly acts synergistically with TAJ/TROY in 293T cell killing, functioning as a positive regulator rather than an initiator. To determine whether enhanced cytotoxicity occurs through caspase-3-mediated proteolysis, we analysed the activation of caspase-3 in TAJ/TROY-transfected or TAJ/TROY- and
PDCD5 enhances TAJ/TROY induced paraptosis

As shown in Fig. 5B, DEVD cleavage activity in all treated cells was similar to that observed in mock-transfected cells. Our results suggest that caspase activity is not involved in the death enhancement of PDCD5.

Upregulation of PDCD5 protein in TAJ/TROY-transfected cells

We have demonstrated previously (Liu et al., 1999) that withdrawal of growth factor induced an increase in PDCD5 protein in apoptotic TF-1 cells. To determine whether PDCD5 protein expression is enhanced in response to TAJ/TROY overexpression, we used an FITC-labeled anti-PDCD5 monoclonal antibody to assess protein levels in treated 293T cells. Using a FACScalibur flow cytometer, we showed that endogenous PDCD5 in TAJ/TROY-overexpressing cells was significantly upregulated at 30 hours after transfection compared with mock-transfected and PDCD5-overexpressing cells (Fig. 6A). Transfection of a Bax-encoding plasmid also induced a relatively weak increase in PDCD5 protein (Fig. 6A).

Discussion

Although it is established that caspase-regulated apoptosis is an important form of PCD, recent studies indicate that, in many instances, PCD is caspase independent and non-apoptotic (Crawford and Bowen, 2002; Davidson et al., 2002; Liu et al., 2003; Guegan and Przedborski, 2003; Borner and Monney, 1999; Kitanaka and Kuchino, 1999). In this report, we
demonstrate that TAJ/TROY induces a form of non-apoptotic cell death characterized by paraptosis-like morphology. This type of cell death involved extensive cytoplasmic vacuolation, mitochondria swelling, caspase activity deficiency, loss of mitochondrial membrane potential and surface exposure of PS but displayed none of the classical nuclear characteristics of apoptosis. Our results, together with those of Eby et al. (Eby et al., 2000), who previously reported TAJ-induced apoptosis independent of oligonucleosomal DNA fragmentation and caspase activation, provide strong evidence that TAJ/TROY overexpression leads to activation of a cell death program that is totally distinct from apoptosis. This cell death might represent an alternative form of paraptotic PCD, which has been overlooked until now. However, terms such as atypical PCD, non-apoptotic PCD or caspase-independent PCD that have appeared in recent literature are still ambiguous. We assume that paraptosis is one type of caspase-independent PCD among many. The precise classification, nomenclature and description of cell deaths should be re-assessed by scientists involved in this field.

In contrast to apoptotic PCD, in which the central mechanism and molecules involved in the transduction of death signals have been largely delineated, information regarding the regulation of caspase-independent paraptotic PCD is currently limited. Our studies demonstrate that paraptosis-like cell death is accompanied by mitochondria swelling and loss of the ΔΨm, suggesting the involvement of mitochondrial products in this death pathway. Ultrastructural characteristics revealed that mitochondria lost membrane potential via a swelling way, whereas, in classic apoptosis, the mitochondria maintains its shape and volume. Further studies are required to determine
whether known or unknown proteins localized at the mitochondrial intermembrane initiate an intrinsic paraptosis-like PCD cascade or disruption of mitochondrial electron transport and energy metabolism directly kills the cell.

An early loss of normal cell membrane asymmetry with translocation of PS to the outer leaflet of the phospholipid bilayer membrane is a general feature of apoptosis, regardless of the initiating stimulus (Martin et al., 1995). Sperandio et al. compared apoptosis, paraptosis and necrosis with respect to chromatin condensation, DNA fragmentation, mitochondrial swelling, caspase-3 activity and the effect of apoptosis inhibitors (Sperandio et al., 2000). However, the issue of whether cells undergoing paraptosis display PS translocation to the plasma membrane remains to be clarified. Using annexin-V staining and FACS analysis, we observed PS exposure in TAJ/TROY-transfected cells. Although data on the mechanisms leading to PS externalization in apoptotic cells is limited, it is proposed that depression of the aminophospholipid translocase and calcium-mediated, non-specific flip-flop of phospholipids play a role (Bratton et al., 1997). We speculate that the pathway(s) leading to PS externalization cross-link the cell death programs of apoptosis and paraptosis. Outer leaflet PS is the most extensively characterized signal in tissues for the non-inflammatory engulfment of apoptotic cells (Fadok et al., 1992; Fadok et al., 2001). It is possible that phagocytosis of paraptotic cells by macrophages is additionally mediated by PS. Although annexin-V binding is a widely used method to evaluate apoptosis, our results suggest that cells undergoing paraptosis also display surface exposure of PS. Thus, multiparameter analyses should be used to identify apoptotic or non-apoptotic cells in the future.

Here, we demonstrate for the first time that PDCD5 overexpression enhances caspase-independent, paraptosis-like cell death triggered by TAJ/TROY. We cloned the novel PDCD5, an increased expression gene observed during the apoptosis of TF-1 cells induced by growth factor withdrawal (Liu et al., 1999). Further studies showed that transient or stable overexpression in cell lines or addition of recombinant PDCD5 protein to culture medium facilitates apoptosis triggered by various apoptosis stimuli (Liu et al., 1999). Chen et al. (Chen et al., 2001) reported that the translocation of PDCD5 from the cytoplasm to the nuclei is an early event in the apoptotic process. Rui et al. showed that the transfer of anti-PDCD5 antibody into HeLa cells inhibits etoposide-induced apoptosis (Rui et al., 2002). Decreased levels of endogenous PDCD5 expression by antisense oligonucleotides delayed the apoptosis of Jurkat cells induced by etoposide (data not shown). These observations suggest that PDCD5 plays a significant role in the cell-intrinsic apoptosis program. In this regard, an interesting question is whether PDCD5 is involved in non-apoptotic PCD under either pathophysiological or physiological conditions. The finding that not only is PDCD5 upregulated during the paraptotic process triggered by TAJ/TROY overexpression but it also acts synergistically with TAJ/TROY on 293T cell death implicates PDCD5 as a potent regulator of this paraptosis-like death. Because mechanistic knowledge associated with paraptosis is currently limited, identification of genes that participate in paraptotic cell death might enhance our understanding of this process. This preliminary study showed that caspase-3 activity is not involved in the parapotic death enhancement by PDCD5 (Fig. 5B). Future studies are required to elucidate the mechanism of the promoting activity of PDCD5.

The relationship between apoptotic and paraptotic PCD remains to be clarified. Out of 7075 differentially expressed genes, less than 2% are common to both cell death programs (Sperandio et al., 2000). We propose that differentially expressed genes are responsible for the distinct cell death pathways. So, PDCD5 might be one of the key molecules connecting the two cell death programs. Programmed cell death in Dictyostelium discoideum (Cornillon et al., 1994; Levraud et al., 2003) and Schizosaccharomyces pombe (Jurgensmeier et al., 1997) also feature cytoplasmic vacuolation, suggesting that paraptosis is an ancient form of PCD. Because PDCD5 shares significant sequence homology with the corresponding proteins of several species (including from archaea to mammals), we predict that this protein and its orthologs are core regulators of PCD in phylogenetically old metazoa, unicellular eukaryotes as well as vertebrates.

In conclusion, our findings suggest that TAJ/TROY overexpression induces parapoptosis-like cell death and that PDCD5 plays a promoting role in this process. Biochemical and genetic experiments that aim to clarify the molecular mechanisms of paraptotic PCD should shed light on carcinogenesis, neurodegeneration, development and evolutionary aspects of cell death programs.

We thank Y. S. Gao for the DNA sequencing, B. H. Hu, X. Chang, X. R. Zhao and H. H. Hao for technical assistance with the electron microscope, and Elixigen (Shanghai) for assistance in the revision of the manuscript. This work was supported by a grant from the National Natural Science Foundation of China (39870427) and the National High Technology Research and Development Program of China (2002BA711A01).

References

Baker, S. J. and Reddy, E. P. (1998). Modulation of life and death by the TNF receptor superfamily. Oncogene 17, 3261-3270.

Borning, C. and Monney, L. (1999). Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ. 6, 497-507.

Bratton, D. L., Fadok, V. A., Richter, D. A., Kailey, J. M., Guthrie, L. A. and Henson, P. M. (1997). Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. J. Biol. Chem. 272, 26159-26165.

Castro-Obregon, S., Del Rio, G., Chen, S. F., Swanson, R. A., Frankowski, H., Rao, R. V., Stoka, V., Vesce, S., Nicholls, D. G. and Bredesen, D. E. (2002). A ligand-receptor pair that triggers a non-apoptotic form of programmed cell death. Cell Death Differ. 9, 807-817.

Chen, Y., Douglass, T., Jeffes, E. W., Xu, Q., Williams, C. C., Arapjirakul, N., Delgado, C., Kleinman, M., Sanchez, R., Dan, Q. et al. (2002). Living T9 glioma cells expressing membrane macrophage colony-stimulating factor produce immediate tumor destruction by polymorphonuclear leukocytes and macrophages via a parapoptosis-induced pathway that promotes systemic immunity against intracranial T9 gliomas. Blood 100, 1373-1380.

Chen, Y., Sun, R., Han, W., Zhang, Y., Song, Q., Di, C. and Ma, D. (2001). Nuclear translocation of PDCD5 (TFAR19): an early signal for apoptosis? FEBS Lett. 509, 191-196.

Cornillon, S., Foa, C., Davoust, J., Buonavista, N., Gross, J. D. and Golstein, P. (2002). Programmed cell death in Dictyostelium. J. Cell Sci. 107, 2691-2704.

Crawford, K. W. and Bowen, W. D. (2002). Sigma-2 receptor agonists activate a novel apoptotic pathway and potentiate antineoplastic drugs in breast tumor cells. Cancer Res. 62, 313-322.

Davidson, W. E., Haudenschild, C., Kwon, J. and Williams, M. S. (2002). T cell receptor ligation triggers novel nonapoptotic cell death pathways that are Fas-independent or Fas-dependent. J. Immunol. 169, 6218-6230.
Green, D. R. and Reed, J. C. (2000). Tumor necrosis factor receptor family, members in the immune system. *Semin. Immunol.* 10, 423-434.

Li, L., Chen, Y. Y., Zheng, R., Ma, D. L. and Wang, D. B. (2001). Apoptotic protease activating factor 1 (Apaf-1)-independent cell death suppression by Bcl-2. *J. Exp. Med.* 191, 1709-1720.

Headon, D. J. and Overbeek, P. A. (1999). Involvement of a novel TNF receptor homologue in hair follicle induction. *Nat. Genet.* 22, 370-374.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature* 407, 770-776.

Hu, S., Tamada, K., Ni, J., Vincenz, C. and Chen, L. (1999). Characterization of TNFRSF19, a novel member of the tumor necrosis factor receptor superfamily. *Genomics* 62, 103-107.

Jacobson, M. D., Weil, M. and Raff, M. C. (1997). Programmed cell death in animal development. *Cell* 88, 347-354.

Jurgensmeier, J. M., Krajewski, S., Armstrong, R. C., Wilson, G. M., Prehn, J. H. (2002). Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. Role of caspase-3. *J. Biol. Chem.* 277, 24506-24514.

Rehm, M., Dussmann, H., Janiak, C. R. U., Tavares, J. M., Kogel, D. and Prehn, J. H. (2002). Caspase-3 is dispensable for a sublethal form of caspase activation during apoptosis. *J. Biol. Chem.* 277, 15754-15759.

Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M. and Kroemer, G. (1999). Bcl-2 inhibits the mitochondrial release of an apoptotic protease. *Cell* 91, 443-446.

Vaux, D. L. and Korsmeyer, S. J. (1999). Cell death in development. *Cell* 96, 1313-1341.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K. and Takahashi, R. (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol. Cell* 8, 613-621.

Tanabe, K., Nakanishi, H., Maeda, H., Nishioku, T., Hashimoto, K., Liou, S. Y., Akamine, A. and Yamamoto, K. (1999). A predominant apoptotic death pathway of neuronal PC12 cells induced by activated microglia is displaced by a non-apoptotic death pathway following blockade of caspase-3-dependent cascade. *J. Biol. Chem.* 274, 15725-15731.

Thorburn, N. A. and Lazebnik, Y. (1998). Caspases: enemies within. *Science* 281, 1312-1316.