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Revival of apoptotic cells that display early-stage dynamic membrane blebbing

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Abstract **The critical point at which apoptosis becomes irreversible and how cells attain an anti-apoptotic state remain unknown. Here, we report that apoptotic cells undergoing early-stage dynamic membrane blebbing revive. We examined this phenomenon in cell lines that stably express 2DED2DD, a modified FADD produced by fusing the tandem death effector domains (DEDs) and tandem death domains (DDs). Induction of apoptosis caused rapid blebbing. Eight hours later, most cells shrunk while some detached from the flask. Twenty-four hours later, when activated caspase 3 decreased, more than half the cells revived and appeared normal, probably due to the induction of unidentified anti-apoptotic proteins.**

Keywords: Apoptosis; FADD; Biotechnology; Membrane blebbing; Anti-apoptotic protein

1. Introduction

Whether a cell dies or lives depends on the balance of proapoptotic and anti-apoptotic factors within multiple and overlapping networks of cellular cascades. Malignant cells are able to survive because they counteract various apoptotic proteins through various means, including strong induction of anti-apoptotic proteins, amplification of anti-apoptotic genes, deletion of apoptotic genes, and depression of apoptotic proteins through epigenesis [1,2].

Fas/TNFRSF6-associated protein with death domain (FADD) is an adaptor molecule that mediates apoptotic cell signals by interacting with various cell surface receptors [3]. Through its C-terminal death domain (DD), FADD can be recruited by different tumor necrosis factor receptors (TNFRs) such as Fas/TNFRSF6 receptor, TNFRSF25, and TNFSF10/TRAIL receptor. The FADD N terminus contains a death effector domain (DED), which recruits caspase to the death-inducing signaling complex (DISC) and initiates the apoptotic caspase cascade [4]. Recruitment of caspase 8 to the receptors results in oligomerization of the caspase 8 protein, which then drives its autoactivation through self-cleavage. Finally, activated caspase 8 activates other downstream caspases.

Previously, we showed that modification of FADD to 2DEDplusE by fusing its tandem DEDs to the E protein of lambda phage, a head coat protein with self-assembly activity, greatly increases the apoptosis-inducing activity of FADD in both adherent NIH3T3 and HEK293 cells [5]. In that study, we also showed that 2DED2DD protein, which is composed of tandem DEDs and tandem DDs, displays intermediate apoptosis-inducing activity. The apoptosis-inducing activity of 2DED2DD is stronger than that of unmodified FADD but weaker than that of the 2DEDplusE protein. Five hours after induction, approximately 40% of cells were affected and had detached. Interestingly, approximately 24 h after induction of 2DED2DD expression, the number of adherent cells increased again. It is possible that this increase was due to reattachment of cells. An alternative but less likely possibility is that this increase was due to proliferation of surviving cells. In the present study, we analyzed in detail this phenomenon – revival of apoptotic cells – because this phenomenon is an excellent model for analyzing the mechanism underlying the survival of malignant cells and of normal cells that adopt an anti-apoptotic state through the balance of pro- and anti-apoptotic proteins.

2. Materials and methods

2.1. Live cell imaging by time-lapse microscopy

Cells were observed using phase contrast microscopy (Olympus IX71, Tokyo, Japan) with an UplanFL N40×/0.75 Ph objective lens and a CO_2 incubator on a heated stage for microscopy (Olympus MI-IBC-I, Japan). Images were recorded using a CCD camera (Hamamatsu ORCA-ER, Japan) and AQUA-C imaging software (Compix Inc.). Typically, 5×10^4 cells were plated onto 35 mm, poly-D-lysine-coated glass bottom dishes. Twenty-four hours after subculture, tetracycline was added to the culture medium and the cells were recorded continuously at 5 min intervals.

2.2. Immunoblot analysis

Cells were plated at a density of 2×10^5 cells per 35 mm dish with an appropriate DMEM medium. Twenty-four hours after subculture, tetracycline was added. At the indicated times, both detached and attached cells were pooled, and cell extracts were prepared by washing the cells with phosphate-buffered saline (PBS) and adding 50 μl of Chaps Cell Extract Buffer (Cell Signaling Technology, Inc.) or solubilization buffer (25 mM Tris–HCl, pH 6.8; 2% SDS). The protein

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Abbreviations: FADD, Fas-associated death domain protein; DISC, death-inducing signaling complex; cFLIP, cellular FADD-like interleukin-1β converting enzyme-inhibitory protein; PBS, phosphate-buffered saline

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concentrations in the extracts were determined with a BCA kit (Pierce). Forty micrograms of each extract were separated on 10% polyacrylamide gels and blotted onto nitrocellulose transfer membranes (PROTRAN; Schleicher & Schuell, Dassel, Germany) using a semi-dry system (ATTO Co., Tokyo, Japan). Membranes were blocked with 1% blocking reagent (Boehringer) in PBS containing 0.1% Tween-20, then incubated with primary antibody solution prepared in 1% BSA/0.1% Tween-20/PBS overnight at 4 °C. We used the following primary antibodies: mouse monoclonal anti-FADD antibody (1:1000) from Stressgen Bioreagents Corp.; rabbit monoclonal anti-cleaved caspase 3 (Asp175) (5A1) (1:1000), rabbit polyclonal anti-Bcl2 (1:1000), anti-cFLIP (1:1000), and anti-cIAP1 (1:1000) antibodies from Cell Signaling Technology Inc.; and mouse monoclonal anti-actin (Ab-5) antibody (1:5000) from BD Biosciences. After incubation, membranes were washed three times with PBS containing 0.1% Tween-20 and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibody (1:2000; Promega) in 1% blocking reagent in PBS containing 0.1% Tween-20. After washing, immunoreactive bands were detected using the ECL plus Western blotting detection system (Amersham Biosciences) according to the manufacturer's instructions and visualized with a Luminescent Image Analyzer LAS1000 (Fujifilm).

2.3. Construction of plasmids expressing modified FADD-tagged 3×FLAG

The plasmid pTREx-2DED2DD allows production of a foreign protein under the control of a tetracycline repressor system and constitutively produces EGFP from a CMV promoter. pTREx-2DED2DDFLAG was constructed by inserting a 3×FLAG sequence into the C terminus of the 2DED2DD open reading frame. All constructs were confirmed by DNA sequencing.

2.4. Fluorescence microscopy analysis

The cells were plated in appropriate medium on poly-D-lysine-coated glass bottom dishes. Twenty-four hours after subculture, tetracycline was added. At the indicated times, the cells were washed with PBS, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer and 3.4% sucrose for 30 min at room temperature. The cells were then incubated with blocking solution (10% goat serum, 1% BSA in PBS) for 1 h at room temperature and incubated with primary antibodies (anti-cleaved caspase 3 (Asp175) (5A1) or anti-FADD) in 1% BSA/PBS overnight at 4 °C. After washing, the cells were incubated with Cy3-conjugated anti-rabbit or anti-mouse IgG antibodies (Amersham). The cells were observed using an inverted microscope (Zeiss Axiovert S100, Germany; lens Plan-NEOFLUAR 10×/0.30 and Plan-NEOFLUAR 40×/0.75) and recorded using a CCD camera (Olympus DP70, Japan).

3. Results and discussion

3.1. Apoptotic cells were revived

In our previous study, we reported that cells expressing 2DED2DD protein detached from glass dishes, but one day after inducing 2DED2DD expression, the number of adherent cells increased, perhaps due to cell reattachment or due to other reasons ([5] and cf. Fig. 3A). In the present study, we analyzed this phenomenon in detail to determine whether apoptotic cells can be revived. We studied this phenomenon in the stable cell line of HEK293 cells we used previously because this line allows us to induce 2DED2DD expression, which is under the control of a tetracycline repressor system [5]. As shown in Fig. 1, immediately after treating the cells with tetracycline, the cells moved actively like normal cells. Sometimes we observed cells dividing. Five hours after inducing 2DED2DD expression, some cells shrunk and underwent membrane blebbing. Hours (8–10) after induction, most cells became spherical and some cells detached from the bottom of the dish. Most cells shrunk and formed clustered small balls, that is, exhibited blebbing.

From our time-lapse microscopy observations, we found that cells expressing 2DED2DD have three fates. Firstly, some cells initially showed signs of apoptosis then appeared normal. Shortly after 2DED2DD induction, these cells shrunk and displayed membrane blebbing. Curiously, the blebbing disappeared then re-appeared repeatedly a couple of times. Twenty-four hours after induction, the blebbing faded completely (Fig. 1, arrowhead), and the cells bound tightly to the

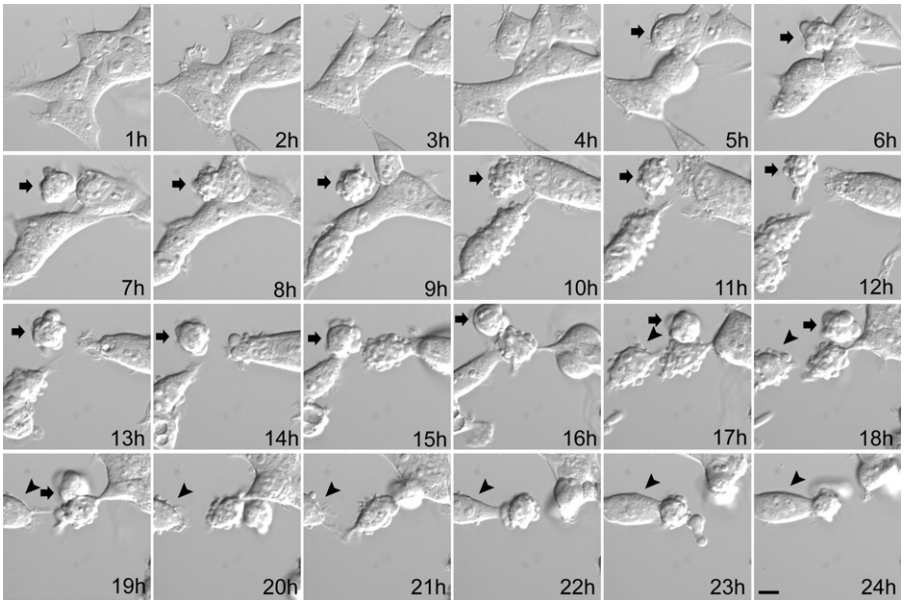

Fig. 1. Serial images of apoptotic 2DED2DD-expressing cells that were revived. A stable cell line expressing 2DED2DD was plated onto poly-D-lysine-coated glass bottom dishes. One day after plating, tetracycline was added to the culture medium. Serial images of 2DED2DD-expressing cells were acquired by time-lapse microscopy at intervals of 60 min. A movie showing the movement of these cells at intervals of 5 min is available as Supplementary material. Scale bar: 10 μm.

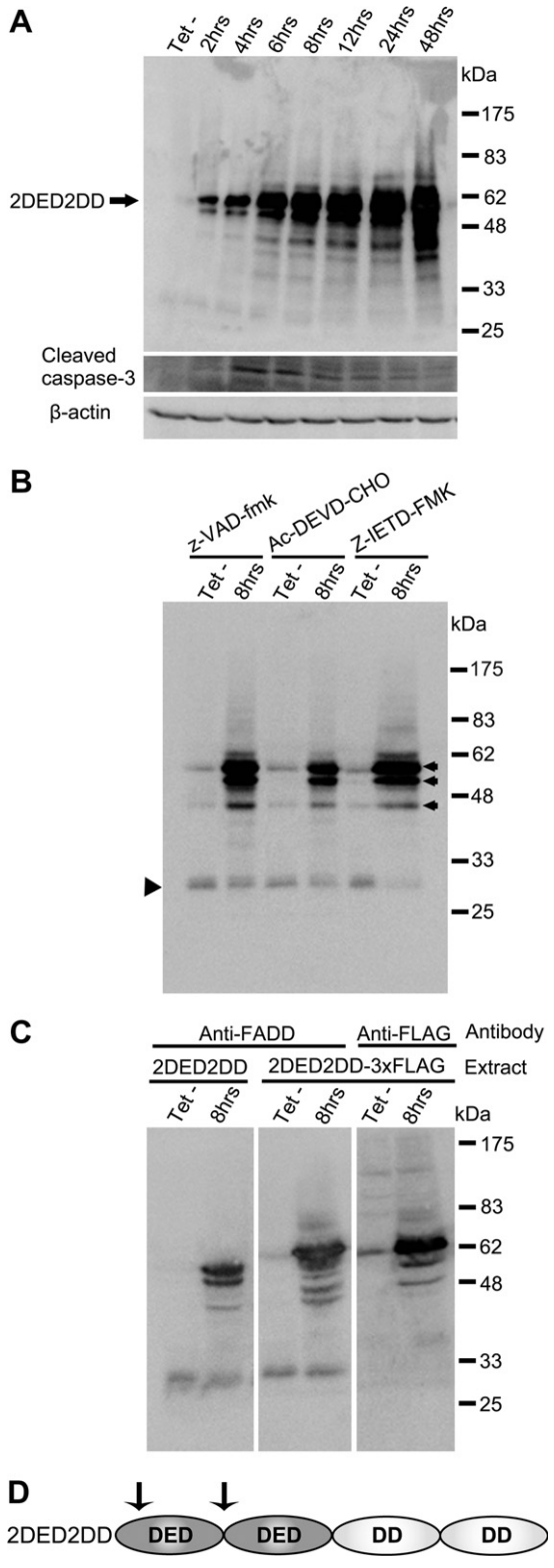

Fig. 2. Time course of the induction of 2DED2DD expression and activation of caspase 3. (A) Immunoblot analysis was performed using extracts of 2DED2DD-stably transfected cells 2, 4, 6, 8, 12, 24, and 48 h (lanes 2–8) after tetracycline induction. Extract from cells cultured without tetracycline is shown in lane 1. Activated caspase 3 was detected using anti-caspase 3 antibody and the ECL plus Western blotting detection system. (B) The cells were treated with z-VAD-fmk, Ac-DEVD-CHO, or z-IETD-fmk 2 h before the addition of tetracycline. Arrowheads point to a full-length form and two processed forms of 2DED2DD. The triangle indicates endogenous FADD. (C) C-terminal 3 × FLAG-tagged 2DED2DD was produced in HEK293 cells. Immunoblot analysis was performed on extracts from these cells 8 h after induction with tetracycline. The immunoblots were probed with either anti-FADD or anti-FLAG tag antibodies. (D) Schematic diagram showing the domain organization of modified FADD, 2DED2DD protein, and its putative processed sites. DED and DD represent death effector domain and death domain, respectively. Arrows point to the putative processed sites of 2DED2DD.

surface of the dish and spread normally. Secondly, some cells that displayed membrane blebbing detached from the dish and died (Fig. 1, arrow). The death of these cells was confirmed by propidium iodide (PI) staining. Thirdly, some cells initially showed very few signs of apoptosis then appeared normal. Upon 2DED2DD induction, these cells shrunk but did not display membrane blebbing. Shortly thereafter the cells spread like typical healthy cells and moved actively. Although the fates of individual cells expressing 2DED2DD varied, overall most cells began to shrink between 8 h and 12 h after induction, underwent membrane blebbing, then spread normally as they did 24 h before induction. No apoptotic cells were observed in a control experiment performed under the same conditions but without tetracycline (data not shown).

3.2. Membrane blebbing is reversible in cells expressing full or processed 2DED2DD protein

To examine the time course of 2DED2DD protein expression after tetracycline induction relative to the reversal of membrane blebbing, we analyzed by immunoblotting extracts of 2DED2DD stably transfected cells at different times after tetracycline induction. Before adding tetracycline, we detected two faint signals – a 59 kDa band and a 42 kDa band – due to leakage of our expression system. After adding tetracycline, we detected three bands – 59 kDa, 54 kDa, and 42 kDa – with anti-FADD antibody, which recognizes DD of FADD. The 59 kDa band represented full-length 2DED2DD protein, which was confirmed by additional immunoblot analysis and by analyzing products of an in vitro translation/transcription system (data not shown). Intact FADD has a molecular weight of 23 kDa and an apparent molecular weight of 28 kDa on SDS–PAGE (Fig. 2, triangle) [6]. Proteins representing the 54 kDa and 42 kDa bands were processed in vivo, probably during the apoptosis cascade. All three signals increased gradually 2–24 h after induction. The overall expression of 2DED2DD was consistent with the progression of apoptosis.

Immunoblot analysis using antibodies against the active form of caspase 3, antibodies that do not recognize procaspase 3, was performed on the same extracts described above. As shown in Fig. 2B, peak signals representing the active form of caspase 3 were detected 4–6 h after induction, at a time slightly before cells begin to shrink.

To examine whether some caspases participate in processing the 54 kDa and 42 kDa forms of 2DED2DD, we pre-treated 2DED2DD-expressing cells with caspase inhibitors before tetracycline induction. The ratio of the three bands were not affected by z-VAD-fmk, which inhibits all known caspases; Ac-DEVD-CHO, which specifically inhibits caspases 3 and 7; and z-IETD-fmk, which specifically inhibits caspase 8 and Granzyme B. Thus, another non-caspase proteinase appears to be involved in the processing of 2DED2DD. However,

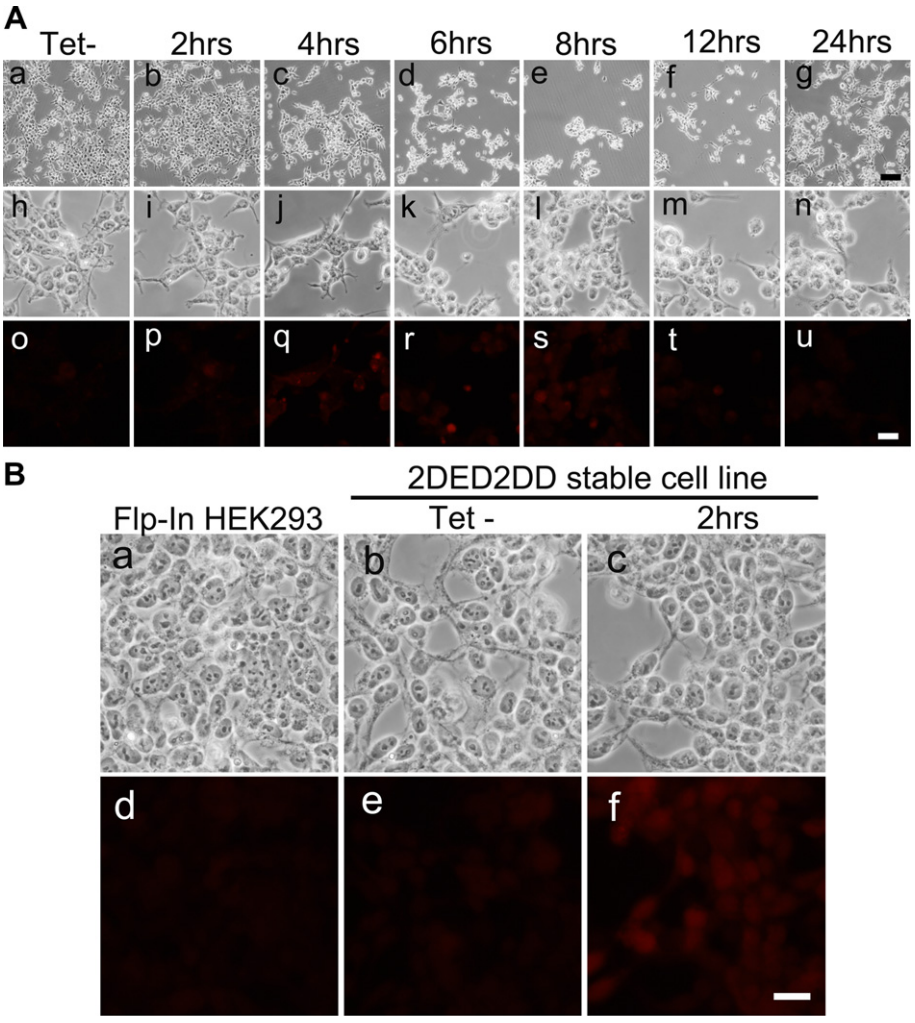

Fig. 3. Uniform activation of caspase 3 after induction of 2DED2DD protein. (A) The stable cell line expressing 2DED2DD was subcultured in poly-D-lysine-coated glass bottom dishes. At the indicated times after tetracycline induction, the cells were fixed with 4% paraformaldehyde and immunostained with anti-caspase antibodies that recognize the processed, active form of caspase 3, followed by Cy3-labeled secondary antibodies. The cells were observed using fluorescence microscopy. (a–g) Low magnification phase contrast microscopy images. Scale bar: 100 μm. (h–n) High magnification phase contrast microscopy images. (o–u) Fluorescence microscopy images of cells stained with anti-caspase 3/Cy3-labeled antibodies. Scale bar: 25 μm. (B) Two hours after tetracycline induction, cells were fixed then sequentially immunostained with anti-FADD antibodies and Cy3-labeled secondary antibodies. Phase contrast (a–c) and fluorescence (d–f) microscopy images of untreated cells (a–e) and tetracycline-treated cells (c, f). Scale bar: 25 μm.

despite our caspase inhibitor results, we cannot exclude completely that caspase 8 or 10 processes 2DED2DD by means of the physical binding of the two DEDs of caspase 8/10 to the tandem DEDs of 2DED2DD. To analyze in detail the processed sites of 2DED2DD, we fused a 3×FLAG tag to the C terminus of 2DED2DD protein, transiently expressed the fusion protein in HEK293 cells, and detected the protein by immunoblotting with anti-FLAG antibodies. As expected, in the cell extracts we detected three bands representing fragments of FLAG-tagged 2DED2DD protein. These bands corresponded to the 59 kDa, 54 kDa, and 42 kDa bands of full-length and processed 2DED2DD we identified earlier with anti-FADD antibodies, but these bands were 5 kDa larger because of the 3×FLAG tag. These results clearly show that the N-terminal DED domain of 2DED2DD was excised or processed out. By comparing the apparent sizes of the bands on SDS–PAGE with those of the products prepared from an in vitro transcription/translation system (data not shown), we concluded that the 54 kDa fragment was composed of two-thirds of a DED, one complete DED, and two DDs, and the 42 kDa fragment was composed of almost one DED and two DDs (Fig. 2D). Concerning the in vivo processing of original FADD, Zhang and Winito described different types of processed FADD: A proteolytically processed FADD, which is present only in the Fas-associated complex, and phosphorylated and non-phosphorylated forms of original FADD [6]. The physiological functions of these processed forms of FADD remain unknown.

Both z-VAD-fmk, which inhibits all known caspases, and z-IETD-fmk, which specifically inhibits caspase 8, abolished the apoptosis-inducing properties of 2DED2DD. Ac-DEVD-CHO, which specifically inhibits caspases 3 and 7, partially inhibited the apoptosis-inducing properties of 2DED2DD such that only 10% of the cells exhibited apoptosis. These observations strongly suggest that blocking caspase 8 activity during the initial stages of 2DED2DD-mediated apoptosis

induction blocks all apoptotic reactions that occur after caspase 8 activation. Thus, 2DED2DD-mediated apoptosis induction follows the standard caspase cascade.

3.3. Induction of 2DED2DD uniformly activated caspase 3 in all cells

Based on caspase-Glo 3/7 assay results, we previously reported that caspase 3 becomes activated about 3 h after inducing 2DED2DD expression with tetracycline [5], which was confirmed in the present study (Fig. 2A). To confirm whether caspase 3 was activated uniformly in all cells by inducing 2DED2DD, we immunostained cells for the active form of caspase 3 at different times after tetracycline treatment and examined the staining with fluorescence microscopy (Fig. 3A). All cells displayed uniform activated-caspase 3 immunoreactivity, with the most brilliant signals observed 4 and 8 h after induction. This observation indicates that the three fates of cells we described above do not result from variable activated-caspase 3 content in 2DED2DD-expressing cells. The signals representing the active form of caspase 3 decreased gradually from 24 to 42 h after induction, nearly disappearing 72 h after induction. Twenty-four or more hours after induction, despite abundant 2DED2DD expression, the amount of activated caspase 3 decreased uniformly in all cells, with most of the cells taking on a normal appearance. The time course of caspase 3 activity, as determined through a caspase 3 colorimetric assay (data not shown), corroborates our results from Western blotting (Fig. 2A) and immunostaining experiments using an antibody against the active form of caspase 3 (anti-cleaved caspase 3 antibody) (Fig. 3A) . The expression of 2DED2DD in all cells after tetracycline induction was also confirmed by immunostaining with anti-FADD antibody (Fig. 3B). These phenomena strongly suggest, as a means of cellular defense, at certain levels 2DED2DD may trigger the machinery responsible for decreasing activated caspase 3 before apoptosis becomes irreversible.

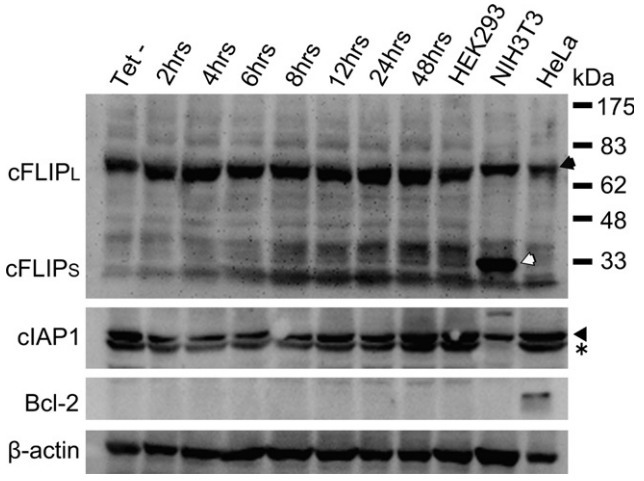

Fig. 4. cFLIP, IAP, and Bcl-2 do not contribute to the revival of apoptotic cells and to the decrease of activated caspase 3 after 2DED2DD induction. Immunoblot analysis was performed using extracts of 2DED2DD-stably transfected cells 2, 4, 6, 8, 12, 24, and 48 h after tetracycline induction. Extracts from cells cultured without tetracycline are also shown. Extracts of parental HEK293, NIH3T3, and HeLa cells were used for control experiments. We analyzed the expression of potential anti-apoptotic proteins with anti-cFLIP, anti-cIAP1, and anti-Bcl2 antibodies and the ECL plus Western blotting detection system. The asterisk indicates a non-specifically immunostained band, based on information provided by the antibody supplier.

To further assess apoptotic blebbing, we stained tetracycline-induced, detached 2DED2DD-expressing cells with Annexin V, which detects the externalization of phosphatidylserine from the inner face of the plasma membrane to the cell surface, and counterstained the cells with DAPI, which detects DNA chromatin condensation (data not shown). Annexin V and DAPI staining indicated that at least two stages of apoptotic blebbing exist. Most cells that displayed early-stage apoptotic blebbing exhibited no or weak Annexin V staining and no DNA chromatin condensation. However, cells that displayed late-stage apoptotic blebbing strongly stained with Annexin V and exhibited significant DNA chromatin condensation. We believe that the cells displaying early-stage apoptotic blebbing are the only cells that can be revived.

To examine the cellular mechanisms of anti-apoptosis processes, we determined whether representative anti-apoptotic proteins, cellular FADD-like interleukin-1β converting enzyme-inhibitory protein (cFLIP), IAP, and Bcl2 [7,8], were induced in our experimental system. As shown in the immunoblots of Fig. 4, Bcl2 was not induced, and the amount of cFLIP and IAP remained unchanged following the induction of 2DED2DD expression. These results indicate that another protein or mechanism is responsible for reversing the blebbing displayed by apoptotic cells.

It is noteworthy that, at least under the apoptotic conditions of our cell systems, HEK293 cells were more resistant to apoptosis than NIH3T3 and HeLa cells. Nevertheless, the amount of cFLIP, IAP, and Bcl2 proteins in HEK293 cells was almost the same or relatively less than that in NIH3T3 and HeLa cells. Thus, it is likely that another anti-apoptosis mechanism is mainly at work in HEK293 cells. One potential mechanism for decreasing the active form of caspase 3 is via the ubiquitin-proteasome pathway. An alternative mechanism may occur through an unknown anti-apoptotic protein induced by 2DED2DD.

The sequential events that occur during apoptosis are as follows: caspase activation, cell shrinkage, dynamic membrane blebbing, condensation of chromatin, and DNA fragmentation. Membrane blebbing is deeply related to nuclear constrictions leading to nuclear fragmentation [9]. Many reports describe the biochemical players involved in membrane blebbing, including the actin-myosin system, the Rho effector protein ROCK I, LIM-kinase 1, and prostate-derived sterile 20-like kinase 2 (PSK2) [10–12]. Apoptosis and apoptotic blebbing, once initiated, were considered to be irreversible despite removal of apoptotic stimuli, although Torgerson and McNiven found that suprastimulation of pancreatic acinar cells reversed blebbing [13]. In the present study, we showed that the apoptotic membrane blebbing that occurs after caspase 3 activation is reversible. That is, apoptotic cells in the initial stages of apoptosis as manifested by membrane blebbing can be revived. The revival of apoptotic cells in our 2DED2DD system can shed light on novel anti-apoptotic mechanisms, because this system employs an artificial recombinant protein and lacks the various side effects that accompany the use of chemical inhibitors. Candidate apoptotic factors will be identified by future proteome analyses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.08.033.

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