Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis

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Detachment of most untransformed adherent cells from the extracellular matrix promotes apoptosis, in a process termed anoikis [1,2]. The death signalling mechanisms involved in this process are not known, although adhesion or transformation by *ras* oncogenes have been shown to protect epithelial cells from apoptosis through activation of phosphatidylinositol 3-kinase and protein kinase B (PKB/Akt) [3]. Here we show that detachment-induced apoptosis (anoikis) is blocked by the expression of a dominant-negative form of FAS-associated death domain protein (FADD) in a number of untransformed epithelial cell lines. Because the soluble extracellular domains of the death receptors CD95, DR4 and DR5 failed to block anoikis, we conclude that ligand-dependent activation of these death receptors is not involved in this process. Detachment induced strong activation of caspase 8 and caspase 3. Detachment-induced caspase-8 activation did not require the function of downstream caspases but was blocked by overexpression of the anti-apoptotic proteins Bcl-2 or Bcl-X_L. We propose that caspase-8 activation is the initiating event in anoikis, which is subsequently subject to a positive-feedback loop involving mitochondrial events.

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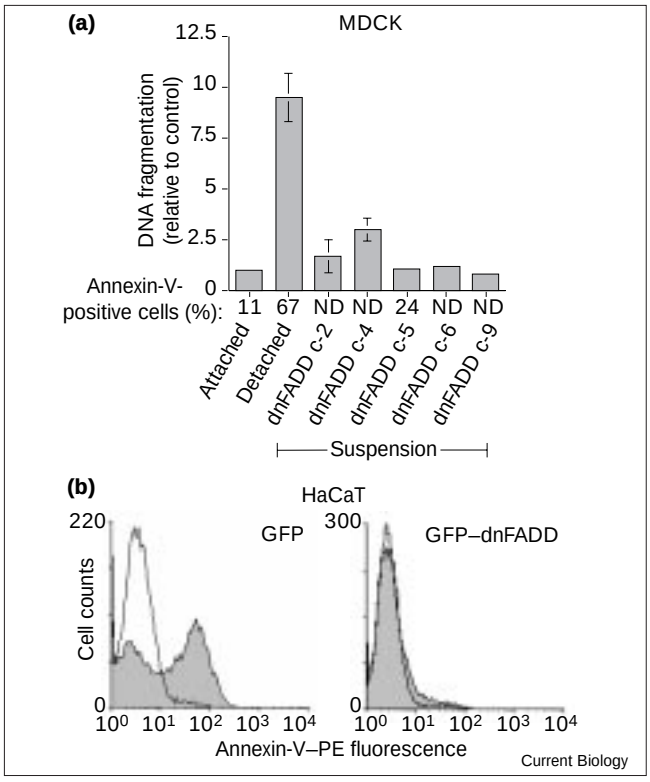
Results and discussion

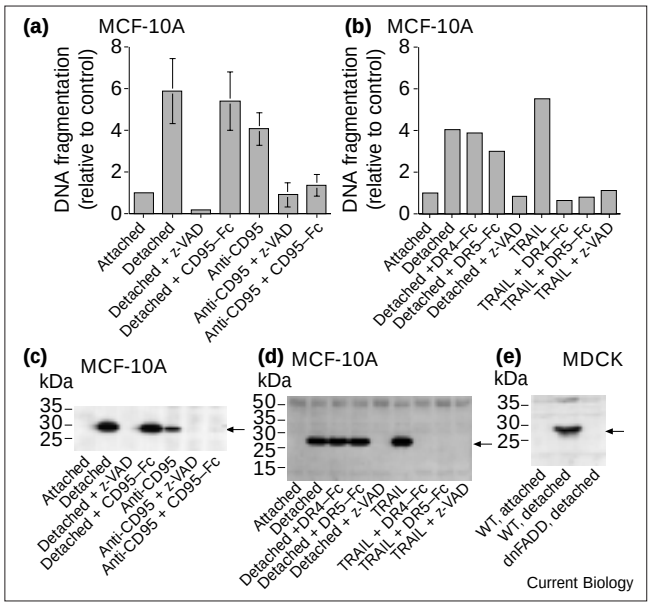
Death receptors and their associated adaptors, such as FADD, and caspases, such as caspase 8, have been implicated in diverse forms of apoptosis (reviewed in [4]). In order to investigate the possible involvement of death-receptor-induced signalling in anoikis, we used a dominant-negative form of FADD (dnFADD), which contains the death domain but not the death-effector domain. As death domains are able to self-associate, dnFADD was able to interact with other death-domain-containing proteins but was unable to activate caspase 8. Madin-Darby canine kidney (MDCK) cells were transfected with the dnFADD expression construct [5] and a number of stable cell lines generated. Following detachment from extracellular matrix for 8 hours, five different clones expressing dnFADD were all protected from apoptosis, as measured using a DNA-fragmentation enzyme-linked immunoabsorption assay (ELISA) (Figure 1a). As DNA fragmentation does not necessarily correlate with apoptosis, these results were verified by staining for annexin V, which indicates the exposure of phosphatidylserine at the outer leaflet of the plasma membrane, an event characteristic of early apoptotic cells. The percentage of cells

Figure 1



Stable expression of dnFADD inhibits anoikis. (a) Apoptosis in five clones (c-2, c-4–6, c-9) of MDCK cells expressing dnFADD was followed by DNA-fragmentation ELISA after cells had been kept in suspension for 8 h. Cells that remained attached to the matrix were used as a control. The percentage of annexin-V-positive cells was determined by fluorescence-activated cell sorter (FACS) analysis. ND, not determined. (b) HaCaT cells stably expressing GFP or GFP–dnFADD were immunostained using phycoerythrin-conjugated Annexin-V (annexin-V–PE). Attached cells, unshaded curves; detached cells, shaded curves. Cells were kept in suspension for 16 h before FACS analysis.

Figure 2



Apoptosis and caspase-8 activation induced by detachment, an anti-CD95 antibody or TRAIL. (a) MCF-10A cells were kept in suspension (detached) or treated with 500 ng/ml anti-CD95 antibody (APO 1–3) for 24 h in a 1:1 mixture of DMEM and HAMS nutrient mixture F12 supplemented with 5% horse serum. In columns 3, 4, 6 and 7, cells were simultaneously treated with either 100 μM z-VAD-fmk or 3 μg/ml soluble recombinant CD95 (CD95–Fc). (b) MCF-10A cells were kept in suspension or treated with 100 ng/ml TRAIL using the same conditions as described in (a) except that some cells were simultaneously treated with either 100 μM z-VAD-fmk or 100 ng/ml soluble recombinant DR4–Fc or DR5–Fc. (c,d) Cells were treated as in (a,b) and the cleaved p20 subunit (arrow) of activated caspase 8 was detected by immunoblotting. (e) Detachment-induced activation of caspase 8 in wild-type (WT) and dnFADD-expressing MDCK cells after 8 h in suspension.

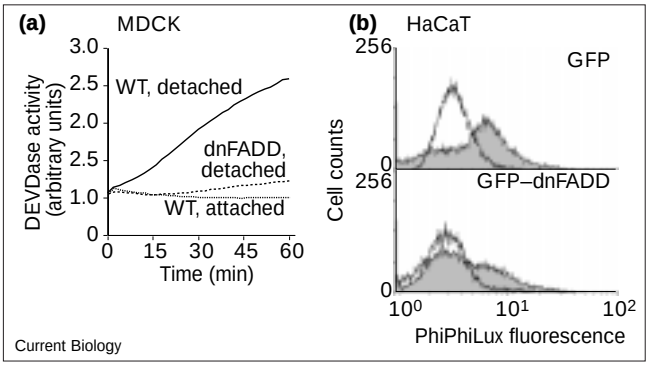
with annexin-V-positive staining is shown in Figure 1a. A similar inhibition of anoikis was seen in the normal immortalised human keratinocyte cell line HaCaT expressing the death domain of FADD fused to GFP (GFP–dnFADD) (Figure 1b).

The inhibition of anoikis by dnFADD raised the possibility that death-receptor signalling could be involved in this process. The normal immortalised human breast epithelial cell line MCF-10A undergoes both CD95-induced and detachment-induced apoptosis (Figure 2a). To study the possible involvement of CD95 ligand (CD95L) in anoikis, we used the soluble recombinant CD95 extracellular domain fused to the immunoglobulin Fc domain (CD95–Fc), which inhibits apoptosis by acting as a soluble decoy receptor [6]. As expected, CD95–Fc was able to block apoptosis induced by an anti-CD95 antibody. CD95–Fc failed to protect the cells from anoikis (Figure 2a), however, suggesting that CD95L engagement of CD95 is not required for this form of cell death. To further investigate the involvement of other death receptors in anoikis, we studied the effects of tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors-1 and -2 (also known as DR4 and DR5, respectively). Soluble recombinant DR4 and DR5 extracellular domains fused to immunoglobulin Fc (DR4–Fc and DR5–Fc, respectively) were similar to CD95–Fc in that they were not able to block anoikis in MCF-10A cells (Figure 2b), although they did prevent TRAIL-induced apoptosis. Similar results were seen in MDCK cells, although in these cells, TRAIL required the inhibition of protein synthesis by cycloheximide to induce apoptosis (see Supplementary material).

We conclude that any possible involvement of death receptor pathways in anoikis suggested by the effects of dnFADD is independent of CD95L and TRAIL interactions with their receptors. This does not, however, rule out the possibility of ligand-independent activation of these receptors, because CD95–Fc, DR4–Fc, and DR5–Fc are only able to block ligand-mediated receptor activation. In addition, TNFα was not able to induce death in MCF-10A or MDCK cells in the presence or absence of cycloheximide, suggesting that the TNF receptor is unlikely to be involved in anoikis (data not shown). At present, we cannot rule out the possibility that detachment induces activation of CD95 or other death receptors in a ligand-independent manner. It is also possible that other death receptors, such as DR3 or DR6, could be involved, in either a ligand-dependent or ligand-independent manner.

Activation of CD95 leads to the formation of a multi-protein death-inducing signalling complex (DISC) to which pro-caspase-8 is recruited and activated [4]. When MCF-10A cells were kept in suspension or treated with an anti-CD95 antibody the activated cleavage product of caspase 8 (p20) became visible by western blotting (Figure 2c). But, if the cells were simultaneously treated with 100 μM of the broad specificity caspase inhibitor z-VAD-fmk, caspase 8 was not activated. This correlated with its effect on DNA fragmentation (Figure 2a). Soluble CD95–Fc blocked caspase-8 activation induced by the anti-CD95 antibody but not that induced by detachment (Figure 2c). When apoptosis of MCF-10A cells was induced by treatment with TRAIL, a similar caspase-8 activation was observed (Figure 2d). Furthermore, DR4–Fc and DR5–Fc were able to block the TRAIL-induced activation of caspase 8. TRAIL-induced apoptosis and caspase-8 activation were also inhibited by z-VAD-fmk. DR4–Fc and DR5–Fc had no effect on detachment-induced activation of caspase 8, however, which is similar to their lack of effect on detachment-induced DNA fragmentation (Figure 2b,d). Similar effects were seen in MDCK cells treated with TRAIL plus cycloheximide (see Supplementary material).

Figure 3



Detachment-induced activation of caspase 3 in MDCK and HaCaT cells. (a) The presence of caspase-3-like activity in cytoplasmic extracts of wild-type (WT) and dnFADD-expressing MDCK cells was measured after 8 h in suspension. The cleavage of a fluorescent substrate z-DEVD-AFC (DEVD) was followed by measuring the increase in the fluorescence intensity. For comparison, the activity in attached wild-type cells is shown. (b) Detachment-induced caspase-3 activation in HaCaT cells expressing GFP (upper panel) or GFP–dnFADD (lower panel). Caspase-3-like activity was measured in attached cells (unshaded curve) or after keeping the cells in suspension for 16 h (shaded curve) by FACS analysis after incubation with a cell-permeable fluorescent substrate, PhiPhiLux-G2D2.

To study the role of FADD in anoikis, we compared caspase-8 activation in wild-type MDCK cells and cell lines stably expressing dnFADD. No detachment-induced caspase-8 activation was seen in cells expressing dnFADD, even though caspase-8 activation was readily detectable in wild-type MDCK cells (Figure 2e). In wild-type MDCK cells, the activation of caspase 8 was visible 3–4 hours after detachment (data not shown). To verify the activation of caspase 8, another antibody was used to detect the first cleavage product of caspase 8 (see Supplementary material).

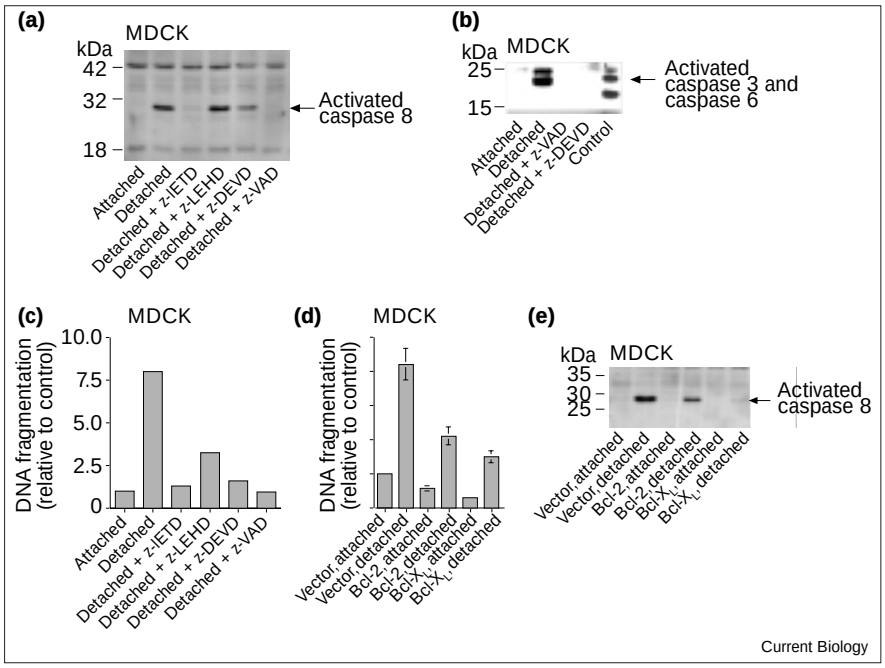
To study the role of FADD in the regulation of downstream, or executioner, caspases, we measured the caspase-3-like activity in MDCK cell lysates by monitoring cleavage of the fluorescent caspase-3 substrate z-DEVD-AFC. No DEVDase activity was seen in attached wild-type MDCK cells, but when cells were detached from matrix and kept in suspension for 8 hours, strong DEVDase activity was seen (Figure 3a). In contrast, cells stably expressing dnFADD did not have any DEVDase activity under these conditions. On the basis of these observations, we conclude that dnFADD acts upstream of the executioner caspases in the death-signalling cascade. To measure the caspase-3-like activity in intact live HaCaT cells, a cell-permeable fluorescent caspase-3 substrate, PhiPhiLux-G2D2, was used. As in MDCK cells, detachment induced activation of caspase-3 in HaCaT cells and this activation was blocked by GFP–dnFADD (Figure 3b). Similarly, caspase 7, like caspase-3, was activated in MDCK cells after detachment (see Supplementary material).

Although the data presented above could be interpreted to mean that caspase-8 activation is an initiating event in anoikis, it is also possible that the strong caspase-8 activation observed occurs as a result of positive feedback from the apoptosome [4] and thus may not initiate apoptosis, but rather might be a consequence of it. In order to investigate this possibility, we examined whether caspase-8 activation following detachment from matrix depends on the activation of other caspases.

Treatment of MDCK cells with 100 μM z-VAD-fmk, a general inhibitor of caspases, resulted in a complete inhibition of anoikis (Figure 4c), and also of detachment-induced caspase-8 and caspase-3 activation (Figure 4a,b). When MDCK cells were treated with 10 μM z-DEVD-fmk, an inhibitor with partial selectivity for caspase 3, however, detachment-induced caspase-3 activation was totally blocked (Figure 4b), as was detachment-induced apoptosis (Figure 4c), but considerable caspase-8 activation still occurred (Figure 4a). Thus, caspase-8 activation can occur independently of caspase-3 activation following detachment from matrix, and thus does not rely on the activation of this downstream executioner caspase. Another possibility is that caspase 8 can be cleaved by caspase 9, so the role of caspase 9 in the activation of caspase 8 was studied. An inhibitor with partial selectivity for caspase-9, z-LEHD-fmk, did not affect detachment-induced caspase-8 activation (Figure 4a), although it inhibited DNA fragmentation (Figure 4c). By contrast, a caspase-8 inhibitor, z-IETD-fmk, blocked both detachment-induced apoptosis (Figure 4c) and caspase-8 activation (Figure 4a). As neither caspase 3 nor caspase 9 appear to be required for caspase-8 activation following detachment from matrix, it seems likely that auto-proteolytic caspase-8 activation is an initiating event in anoikis.

Although the strong activation of caspase-8 detected after cell detachment appears not to require caspase-3 or caspase-9 activity, it could be potentiated by positive feedback involving other post-mitochondrial events. As such events should be inhibited by protection of mitochondria from disruption, the effect of overexpressing Bcl-2 and Bcl-X_L was tested on the induction of apoptosis and caspase-8 activation following detachment from extracellular matrix. As shown in Figure 4d, overexpression of Bcl-2 or Bcl-X_L protected MDCK cells from anoikis, with Bcl-X_L being more effective than Bcl-2. Similarly, Bcl-X_L, and to a lesser extent Bcl-2, also inhibited detachment-induced activation of caspase-8 (Figure 4e). Therefore, it is possible that the activation of caspase 8 seen on detachment is reinforced by mitochondrial damage, perhaps

Figure 4



Effects of the caspase inhibitors Bcl-2 and Bcl-X_L on detachment-induced apoptosis and caspase activation in MDCK cells. Cells were kept in suspension for 8 h before lysis. Either 100 μM z-VAD-fmk (z-VAD), 10 μM z-IETD-fmk (z-IETD), 10 μM z-LEHD-fmk (z-LEHD), or 10 μM z-DEVD-fmk (z-DEVD) were added when cells were detached. (a) Caspase-8 activation was detected by immunoblotting. (b) Affinity labelling of activated caspases with biotin-YVAD-aomk. Control, purified caspases from HL60 cells labelled with Z-EK(biotin)D-aomk (see Supplementary material). (c) Apoptosis measured using DNA-fragmentation ELISA. (d) DNA fragmentation and (e) caspase-8 activation in MDCK cells expressing Bcl-2 or Bcl-X_L after 8 h in suspension.

through caspase-8 cleavage of the Bcl-2 antagonist BID [7,8]. Anoikis could thus be characterised as a type II death receptor signalling pathway [9]. Alternatively, it is possible that Bcl-2 or Bcl-X_L inhibit caspase-8 activation through a mechanism that is independent of effects on the mitochondria: direct interaction of Bcl-X_L with caspase-8 has been reported in overexpression systems [10], but no evidence for this could be found in any of the cells used here (data not shown). Bcl-2 overexpression has also been shown to inhibit formation of the DISC in Jurkat cells (a type II cell line) [11], suggesting the possible involvement of these anti-apoptotic proteins in the earliest death signalling events.

In summary, we propose that detachment of epithelial cells from extracellular matrix induces apoptosis through a pathway involving proteins sequestered by the death domain of FADD, but without the involvement of known ligands for death receptors. Detachment results in caspase-8 activation as an initiating event that leads to the triggering of mitochondrial dysfunction and executioner caspase activation, which result in cell death by apoptosis. A positive-feedback loop from the mitochondria may be involved as Bcl-X_L overexpression inhibits detachment-induced caspase-8 activation.

Supplementary material

Figures showing the effects of apoptosis-inducing agents on the activation of caspases 8 and 7 in MDCK cells, and additional methodological details are available at http://current-biology.com/supmat/supmatin.htm.

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Supplementary material

Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis

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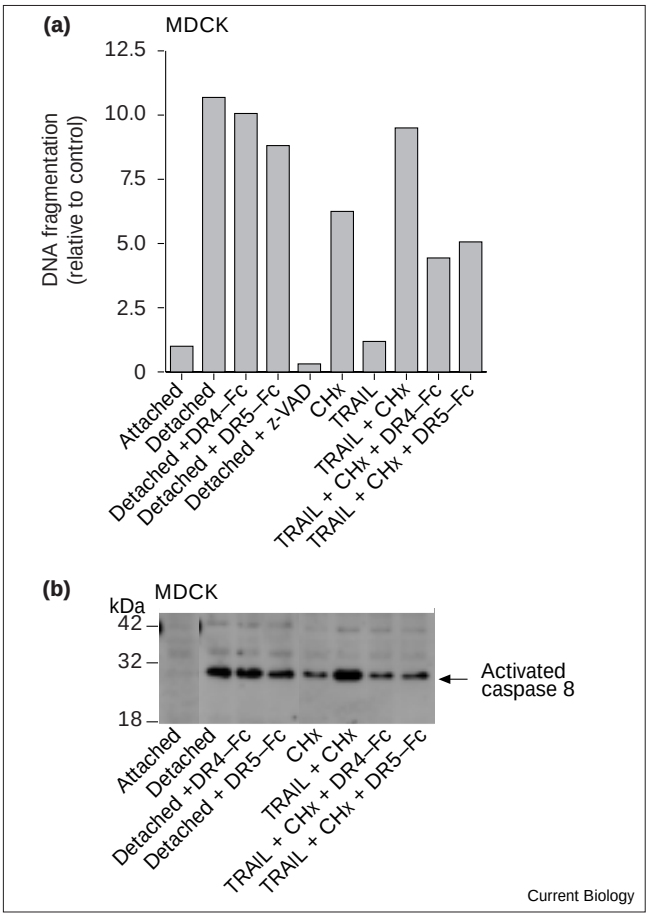
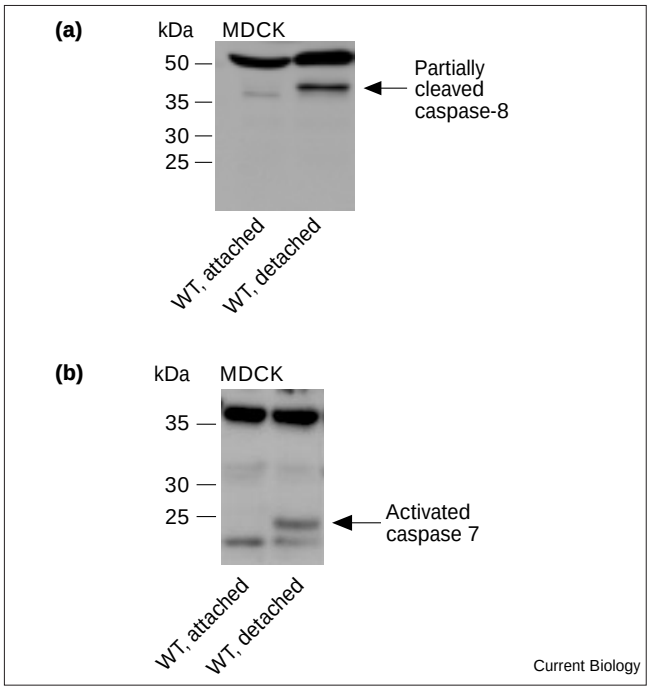
Figure S1



Apoptosis and caspase-8 activation induced by cell–matrix detachment or TRAIL in MDCK cells. DNA-fragmentation ELISA was performed 8 h after detachment or after various treatments. (a) MDCK cells were kept in suspension or treated with TRAIL and/or cycloheximde (CHx) in DMEM supplemented with 10% fetal bovine serum (FBS) for 8 h. Either 100 μM z-VAD-fmk (z-VAD) or 100 ng/ml soluble recombinant DR4 (DR4–Fc) or DR5 (DR5–Fc) were added when cells were detached or treated with 5 μg/ml cycloheximide and 100 ng/ml TRAIL. The increase in DNA fragmentation was calculated by comparing the absorbance of detached cells to the absorbance of wild-type cells maintained on the tissue culture dish. (b) Cells were treated as in (a), lysed with SDS sample buffer, and proteins separated by SDS–PAGE. Caspase 8 was detected by immunoblotting. The arrow indicates the cleaved p20 subunit of activated caspase 8.

Figure S2



Detachment-induced activation of caspase 8 and caspase 7 in MDCK cells. (a) A partially cleaved form of caspase 8 in wild-type MDCK cells was detected by an antibody that specifically recognises this form of caspase 8. Cells were lysed with SDS sample buffer and proteins separated by SDS–PAGE. Caspase 8 was detected by immunoblotting. (b) Detachment-induced activation of caspase 7 in MDCK cells was determined by immunoblotting using an anti-caspase-7 antibody.

Supplementary materials and methods

Materials

Caspase inhibitors were from Calbiochem. PhiPhiLux-G2D2 was from Oncoimmunin. Annexin-V–PE, the anti-CD95 antibody (clone APO 1–3), soluble recombinant CD95 (CD95–Fc) and soluble human recombinant TRAIL were from Alexis. Soluble DR4 (DR4–Fc) and DR5 (DR5–Fc) were from R&D Systems.

Expression vectors

Dominant-negative FADD (dnFADD) was kindly provided by Vishva Dixit (Genentech). Bcl-2 and Bcl-X_L were cloned into pBabe-puro and pBabe-hygro, respectively, and GFP-dnFADD was cloned into pEGFP.

Cell lines

To generate MDCK cells stably expressing dnFADD, and HaCaT cells stably expressing GFP–dnFADD or GFP alone, cells were transfected by lipofection according to manufacturer's instructions (Lipofectamine, GibcoBRL). To generate pools of MDCK cells stably expressing Bcl-2 or Bcl-X_L, MDCK cells expressing the ecotropic retrovirus receptor were infected with ecotropic viruses according to Serrano *et al.* [S1]. The expression of protein was assessed by western blotting of cell lysates.

DNA fragmentation assay

Subconfluent MDCK cells were detached and assayed as described previously [S2]. MCF-10A cells were treated in the same way except

resuspension was in a 1:1 mixture of DMEM and HAMS nutrient mixture F12 supplemented with 5% horse serum. DNA-fragmentation ELISA was performed using the Cell Death Detection ELISA kit (Boehringer Mannheim) according to the manufacturer's instructions. The results represent at least three independent experiments and the increase in DNA fragmentation was calculated by comparing the absorbance of detached or treated cells to the absorbance of wild-type cells maintained on the tissue culture dish.

Caspase-7 and caspase-8 activation

After the indicated treatment, cells were lysed with sample buffer and proteins were separated by 12% SDS–PAGE. Immunoblotting was performed with an anti-caspase-7 (PharMingen) or anti-caspase-8 (C-20, Santa Cruz Biotechnology) antibody, followed by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham).

Caspase-3 activation

For the fluorometric caspase assay, 5×10^5 cells were analysed as described previously [S3]. Affinity labelling of activated caspases was performed as previously described by Faleiro *et al.* [S4] using the broad specificity caspase substrates biotin–YVAD–aomk and Z-EK(biotin)D-aomk; these bind covalently to caspase active sites, introducing a biotin tag. To measure the intracellular caspase-3-like activity in HaCaT cells, the cells were trypsinized and washed with serum-containing medium. The cell pellet was resuspended with 80 μl of 10 μM PhiPhiLux-G2D2 substrate solution (OncoImmunin) in RPMI supplemented with 5% FBS and processed according to manufacturer's instructions.

Annexin V staining

To measure apoptosis by phosphatidylserine exposure, 10^6 cells were treated as indicated in the text. Cells were trypsinized, washed with serum-containing medium, and with 2 ml of Hepes buffer (10 mM Hepes–NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). The cell pellet was resuspended with 1 ml Hepes buffer and 5 μl of Annexin-V–PE (Alexis) was added. After incubation for 30 min at room temperature, avoiding direct light, the samples were analysed by FACS.

Supplementary references

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