Selective Inactivation of a Fas-associated Death Domain Protein (FADD)-dependent Apoptosis and Autophagy Pathway in Immortal Epithelial Cells

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Although evasion of apoptosis is thought to be required for the development of cancer, it is unclear which cell death pathways are evaded. We previously identified a novel epithelial cell death pathway that works in normal cells but is inactivated in tumor cells, implying that it may be targeted during tumor development. The pathway can be activated by the Fas-associated death domain (FADD) of the adaptor protein but is distinct from the known mechanism of FADD-induced apoptosis through caspase-8. Here, we show that a physiological signal (tumor necrosis factor-related apoptosis-inducing ligand) can kill normal epithelial cells through the endogenous FADD protein by using the novel FADD death domain pathway, which activates both apoptosis and autophagy. We also show that selective resistance to this pathway occurs when primary epithelial cells are immortalized and that this occurs through a mechanism that is independent of known events (telomerase activity, and loss of function of p53, Rb, INK4a, and ARF) that are associated with immortalization. These data identify a novel cell death pathway that combines apoptosis and autophagy and that is selectively inactivated at the earliest stages of epithelial cancer development.

INTRODUCTION

Because apoptosis can suppress tumor development, it is sometimes thought that cancer cells are generally resistant to apoptosis, whereas normal cells are sensitive. In fact, cancer cells are closer to their apoptotic threshold than their normal counterparts and often die more easily than normal cells in response to apoptotic stimuli (Evan and Vousden, 2001; Lowe *et al.*, 2004). Apoptosis sensitization in cancer cells occurs because growth-promoting oncogenic events such as Myc expression (Evan and Littlewood, 1998; Evan and Vousden, 2001; Pelengaris *et al.*, 2002), Rb inactivation (Chau and Wang, 2003), E2F activation (Nahle *et al.*, 2002), and cyclin D3 expression (Mendelsohn *et al.*, 2002) raise the levels of apoptotic proteins or make it easier to activate these molecules and thus reduce the threshold at which apoptosis is activated. Activated oncogenes can also sensitize cells to apoptosis by promoting loss of inhibitors of apoptosis that exist in primary cells (Duelli and Lazebnik, 2000). Immortalization and transformation also sensitize cells to nonapoptotic death (Fehrenbacher *et al.*, 2004).

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Abbreviations used: DD, death domain; FADD, Fas associated death domain protein; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

If cancer cells die more easily than their normal counterparts, which cell death pathways are evaded during tumor development? One answer is that cancer cells must remain below the lowered apoptotic threshold for undergoing stress-induced apoptosis that is caused by the oncogenes that drive cell growth. Indeed, it has been suggested that this may be sufficient to cause cancer without any other cellular defects (Green and Evan, 2002). However, this model does not exclude the possibility that there may also be specific cell death pathways that inhibit cancer development in normal cells that are specifically inactivated during tumor development. Such a pathway would be expected to have the unusual characteristics of working in normal cells but not in cancer cells, and signaling proteins and physiological stimuli that activate this kind of pathway should kill normal cells by mechanisms that are selectively inhibited during the transformation process without affecting other cell death pathways.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a promising treatment for cancer that kills tumor cells with little toxicity to normal tissues in preclinical models (LeBlanc and Ashkenazi, 2003). TRAIL induces apoptosis by binding to two receptors (DR4 and DR5) that contain an intracellular death domain (DD). Ligand binding is thought to result in conformational changes that expose a binding surface for the Fas-associated death domain (FADD) adaptor protein (Thomas *et al.*, 2004a,b). FADD also binds to procaspase-8, resulting in caspase-8 dimerization and activation (Boatright *et al.*, 2003; Boatright and Salvesen, 2003; Donepudi *et al.*, 2003), eventually leading to effector caspase activation. This well-established mechanism causes caspase-dependent apoptosis, which can be blocked by caspase inhibitors or a dominant negative version of FADD (FADD-DD also known as FADD-DN) that has an intact DD

but lacks the death effector domain and cannot bind procaspase-8.

We previously identified an alternate method by which FADD, through its DD alone can kill cells (Morgan *et al.*, 2001; Thorburn *et al.*, 2003). FADD-DD-induced death was unexpected because this molecule is a widely used inhibitor of apoptosis and is unusual because it occurs in primary normal epithelial cells but not in tumor cell lines and involves both the activation of caspases through caspase-9 (not caspase-8) and a separate activity that can be blocked by a serine protease inhibitor [4-(2-aminoethyl)benzenesulfonyl fluoride; AEBSF] (Thorburn *et al.*, 2003). Our previous studies raise several questions. Can a physiological stimulus activate the FADD-DD pathway or is it only induced by overexpression? What is the nature of the caspase-independent cell death that occurs in response to FADD-DD? And, when during epithelial cell transformation do cells lose the ability to respond to this pathway? Here, we answer these questions by showing that the FADD-DD pathway can be activated by a physiological signal (TRAIL receptor activation) working through the endogenous FADD protein and that when caspases are inhibited, the pathway does not kill by apoptosis but instead cells die by autophagy. We also identify a specific step in the transformation process (immortalization) when the pathway is selectively inactivated and show that this occurs via a mechanism that is separate from the known activities that occur during immortalization. These data identify a novel programmed cell death pathway involving apoptosis and autophagy that is selectively disrupted at the earliest stages of epithelial cell transformation.

MATERIALS AND METHODS

Cell Culture and Reagents

Isolation and culturing of normal human prostate epithelial cells from tissue samples was performed as described previously (Morgan *et al.*, 2001; Thorburn *et al.*, 2003). Human breast epithelial cells expressing defined transforming proteins were maintained as described previously (Elenbaas *et al.*, 2001). Tumor cell lines were obtained and cultured as recommended by American Type Culture Collection (Manassas, VA). The mouse mammary epithelial cells were cultured as described previously (Medina and Kittrell, 2000). Mice were obtained from the National Cancer Institute Mouse Models of Human Cancer Repository (Frederick, MD). Mammary tissue was isolated from 6- to 8-wk-old virgin mice, minced, and treated with 400 U/ml collagenase for 1.5–2 h and Pronase (1 U/ml) for 20 min. After digestion, epithelial cells were separated in a Percoll gradient and then cultured on collagen-coated plates in supplemented DMEM/F-12 medium with 1% fetal bovine serum. Recombinant human TRAIL was obtained from Calbiochem (San Diego, CA) and used at 100 ng/ml; zVAD.fmk was obtained from Alexis (San Diego, CA) and used at 0.1 mM. 3-methyladenine (3-MA), cycloheximide, and H33258 (bis-benzimidazole, no. 33258; Aventis, Strasbourg, France) were obtained from Sigma-Aldrich (St. Louis, MO) and used at 10 mM, 0.8 μ g/ml, and 10 μ g/ml, respectively. Antibodies for Western blotting experiments were obtained from Cell Signaling Technology (Beverly, MA).

Microinjection, Adenovirus Infection, and Cell Death Assays

Single cell-based microinjection experiments and cell death/survival assays were performed as described previously (Thorburn *et al.*, 2003). Fifty to 100 cells were injected for each plasmid in each experiment. Each injected cell was identified by virtue of its yellow fluorescent protein (YFP) fluorescence, and its fate was determined after incubation for 20 h. Because ~100 cells were injected at a time for each treatment, it was not possible to perform Western blotting to assess the expression level for YFP, YFP-FADD-DD, or the mutant proteins. Expression levels were therefore determined by visually assessing the amount of YFP fluorescence. The injected cell displayed similar levels of fluorescence, indicating that equivalent levels of each protein was compared. The percentage of living flat intact cells (rounded cells were scored as dead) was calculated for each experiment and the mean percentage of survival $\pm$ SD was calculated from at least four separate experiments by using different preparations of cells and plasmids. Survival >100% indicates that the cells grew during the experiment. Adenovirus purifications were performed using

CsCl₂ centrifugation of doxycycline-regulated AdpEYFPc1, AdpEYFPc1-FADD-DD, and Tet repressor adenoviruses together with AdpEYFPc1-FADD-DD point mutant (V108E), which was constructed as described previously (Thorburn *et al.*, 2003). Cells were infected with ~20 plaque-forming units/cell of each virus for 4 h at which time the virus-containing medium was replaced with regular tissue culture medium. Adenoviral gene expression was repressed with 1 μ g/ml doxycycline, and expression was induced by removing doxycycline. These conditions produced >90% infection efficiency as determined by YFP fluorescence. Population-based cell viability assays after adenovirus infection and treatment with TRAIL, and protease inhibitors were performed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTS) assay from Promega (Madison, WI) as described in the manufacturer's instructions. Time-lapse microscopy was performed in an environmental chamber attached to a Zeiss Axiovert S200 microscope by using a 32 $\times$ objective. Images were captured at 15-min intervals by using a Hamamatsu charge-coupled device (Malvern, PA) camera run by Openlab (Improvision, Warwick, United Kingdom) software, saved in QuickTime Movie format, and frames were captured for still images.

Autophagy Assays

For electron microscopy, cells were cultured in 6-cm dishes, treated with TRAIL or infected with FADD-DD-expressing adenoviruses in the presence of the caspase inhibitor zVAD.fmk as indicated on the figure legends and incubated overnight. Cells were fixed with 2.5% phosphate-buffered glutaraldehyde, postfixed in 1% phosphate-buffered osmium tetroxide, embedded in Spurr's resin, sectioned, double stained with uranyl acetate and lead citrate, and analyzed using a Philips 400 transmission electron microscope. For each treatment and control group, 20–50 randomly chosen cells were analyzed for morphological features associated with autophagy. Cells were scored as autophagy positive by using a scoring method described by Yu *et al.* (2004), where cells with <10 vesicles/cell were scored as normal, 10–19 vesicles/cell were scored as mild autophagy, 20–29 vesicles/cell were scored as moderate autophagy, and >30 vesicles/cell were scored as severe autophagy. The histograms show the percentage of cells in each category. The percentage of the total cell area taken up by autophagic vesicles for each randomly chosen cell was determined using Adobe Photoshop software. For analysis of green fluorescent protein (GFP)-LC3 localization, cells were injected with the expression plasmid along with FADD-DD or control expression plasmids, and time-lapse fluorescence microscopy was performed. Still images were captured from the movies.

RESULTS

A Physiological Stimulus Can Activate the FADD-DD Pathway through the Endogenous FADD Protein

We previously made the surprising discovery that the death domain of FADD can kill normal epithelial cells (Morgan *et al.*, 2001) and showed that FADD-DD-induced cell death involves both caspases and an activity that can be inhibited by AEBSF (Thorburn *et al.*, 2003) that cause different morphological phenotypes in the dying cells. To determine whether a physiological stimulus working through the endogenous FADD protein also could activate this pathway, we examined TRAIL receptor signaling. We reasoned that if TRAIL can activate the FADD-DD-dependent pathway, TRAIL-induced death of normal cells should be inhibited only when caspases and serine proteases are blocked simultaneously. In contrast, caspase inhibitors such as zVAD.fmk alone should block TRAIL-induced death in cancer cells.

For these experiments, we treated normal primary human prostate cells (sensitive to FADD-DD) or DU145 prostate cancer cells (insensitive to FADD-DD) with recombinant TRAIL in the presence of low doses of cycloheximide, which inhibited protein synthesis by ~70% (our unpublished data) and was unable to induce cell death by itself (Figure 1A). Cycloheximide treatment was required in both the normal and cancer cells to allow TRAIL-induced cell death. Cell death was monitored by time-lapse microscopy after treatment with zVAD.fmk and AEBSF. TRAIL killed both cell types, and in both cases the morphology of the dying cells was consistent with apoptosis. However, although zVAD.fmk alone was able to block cell death in cancer cells, only the combination of zVAD.fmk and AEBSF could inhibit cell death in normal cells. The caspase inhibitor on its own did,

Figure 1. TRAIL can kill normal and cancerous epithelial cells by different mechanisms. (A) Time-lapse microscopy of normal prostate epithelial cells or prostate cancer cells treated with TRAIL plus AEBSF and zVAD.fmk. TRAIL kills both cell types, but zVAD.fmk alone can protect only the cancer cells; the combination of zVAD.fmk and AEBSF is required to protect normal cells from TRAIL-induced death. (B) The number of dead cells for each time point was determined by counting rounded cells in individual frames for each treatment. Treatment with zVAD.fmk in the normal cells altered the slope of the line, indicating that the caspase-independent cell death response in normal cells occurred more slowly than caspase-dependent cell death. (C) Normal or cancerous prostate cells were treated with TRAIL in the presence of the protease inhibitors and harvested for Western blot analysis of caspase-3 and PARP cleavage. In both cell types, caspase-3 was activated, leading to PARP cleavage, and the caspase inhibitor zVAD.fmk completely blocked the response. (D) MTS assays of TRAIL treated cells were performed. ZVAD.fmk only partially protected normal cells but completely protected cancer cells. The combination of zVAD.fmk and AEBSF completely protected normal cells.

cells. In cancer cells, both the V108E mutant and the wild-type FADD-DD were equally effective at inhibiting TRAIL-induced cell death. In the normal cells, wild-type FADD-DD plus TRAIL led to increased cell death compared with either FADD-DD or TRAIL alone, whereas the V108E mutant completely inhibited TRAIL-induced cell death. These data indicate that FADD-DD functions differently in normal and cancerous prostate cells and can cooperate with TRAIL to increase normal cell death.

however, alter the morphology of the normal cells as they died in response to TRAIL. High doses of zVAD.fmk have nonspecific effects such as inhibition of cathepsin B (Schotte *et al.*, 1999) that have been implicated in apoptosis regulation. Therefore, our data indicate that inhibition of either caspases or such nonspecific targets is sufficient to prevent death of the normal epithelial cells. Inhibition of caspases resulted in cell death that was associated with cell rounding and detachment but little if any membrane blebbing or cellular fragmentation. Quantitation of the number of dying cells for each treatment during the time course of the experiment (Figure 1B) confirmed that zVAD.fmk alone was able to prevent cancer cell death but had only a partial effect in normal cells. Interestingly, the partial inhibition of normal cell death by the caspase inhibitor also displayed different kinetics as demonstrated by the reduced slope of the line in the time course. These data suggest that the preferred mode of death in the normal cells is via caspase-dependent apoptosis and that the cell death that occurs when caspases are inhibited is slower. This conclusion is also supported by the fact that in the absence of inhibitors the morphology of both normal and cancer cells dying in response to TRAIL is consistent with classical apoptosis with membrane blebbing, cell contraction, and fragmentation. To confirm that caspases were inhibited in both cell types by zVAD.fmk, we assessed the processing of caspase-3 and its substrate poly(ADP-ribose) polymerase (PARP) (Figure 1C). In both normal and cancer cells, the caspase inhibitor completely blocked caspase-dependent cleavage, whereas AEBSF had no effect. Similar results were obtained using MTS assays for viability in normal cells or cancer cells treated with TRAIL (Figure 1D). Together, these data indicate that under these conditions, TRAIL can kill normal cells and cancer cells by different mechanisms with normal cells displaying caspase-independent cell death in addition to caspase-dependent effects. In contrast, and in agreement with a large number of published studies in various cancer cell lines, cancer cells die by caspase-dependent apoptosis in response to TRAIL.

If TRAIL can activate the FADD-DD pathway through the endogenous FADD protein, an FADD-DD mutant that cannot cause cell death when it is expressed in normal cells should function as a dominant negative inhibitor of TRAIL-induced apoptosis. Such a molecule will also be unable to activate caspase-8 because it lacks the DED and blocks death

in cancer cells, too. In contrast, the wild-type FADD-DD molecule should cooperate with TRAIL to increase normal cell death through the FADD-DD pathway but inhibit TRAIL-induced cancer cell death because this should occur only through the established caspase-8-dependent pathway. We tested several point mutants and identified a mutant (V108E) that is unable to induce normal epithelial cell death when injected into cells on its own but is able to bind to TRAIL receptors (Thomas *et al.*, 2004a) and can block TRAIL-induced cell death (Figure 2A).

We expressed wild-type FADD-DD or the V108E mutant in a population of cells from a doxycycline-regulated adenovirus and then treated the normal and cancer cells with TRAIL and measured the response by using a population-based cell viability assay. Figure 2B shows that wild-type FADD-DD increased TRAIL-induced death in normal epithelial cells but blocked TRAIL-induced death in cancer cells. In contrast, the V108E mutant blocked TRAIL-induced death in both normal cells and cancer cells. These data suggest that TRAIL can work through the FADD-DD pathway in normal epithelial cells and that this pathway cannot be activated in cancer cells where all TRAIL-induced cell death occurs through the established caspase-8 pathway.

FADD-DD and TRAIL Can Induce Autophagy in Normal Epithelial Cells

Autophagy has been implicated in tumor suppression (Edinger and Thompson, 2003; Qu *et al.*, 2003; Yue *et al.*, 2003; Alva *et al.*, 2004; Gozuacik and Kimchi, 2004) and has been linked to TRAIL-induced epithelial cell death (Mills *et al.*, 2004). We therefore tested whether autophagy occurs in FADD-DD-expressing normal epithelial cells by using transmission electron microscopy (TEM). Normal epithelial cells expressing adenoviral FADD-DD had numerous membrane-bound vesicles often containing organelles and other cellular fragments (Figure 3). In contrast no significant increase in vesicles was found when the cells expressed the V108E FADD-DD mutant that is unable to kill. Similar vesicle formation occurred in normal cells after treatment with TRAIL. Vesicle formation, which is characteristic of autophagy (Gozuacik and Kimchi, 2004), occurred in the presence of the caspase inhibitor zVAD.fmk, indicating that it is separate from the caspase-dependent apoptosis that occurs in

Figure 3. FADD-DD can cause autophagic vesicle formation in normal epithelial cells. (A) Normal primary prostate epithelial cells or DU145 prostate cancer cells were treated with TRAIL or infected with adenoviruses expressing FADD-DD or V108E FADD-DD as indicated and analyzed by TEM. Large numbers of vesicular structures (arrows) were found in normal cells expressing FADD-DD or treated with TRAIL. Normal cells were treated with zVAD.fmk to prevent caspase-dependent signaling from obscuring any caspase-independent effects. Bars, 5 μ m. (B), higher power images of autophagic vesicles from FADD-DD or TRAIL-treated normal prostate cells showing double membranes and cellular debris. Bar, 0.5 μ m. (C) Cell area taken up by autophagic vesicles, indicating that FADD-DD and TRAIL increase the proportion of each normal cell that is vacuolated.

the normal cells expressing FADD-DD or treated with TRAIL and implying that it participates in the caspase-independent arm of the cell death pathway that is induced

by FADD-DD. Consistent with this idea, vesicles were not formed in response to FADD-DD or TRAIL in prostate cancer cells.

Figure 4. FADD-DD-induced autophagy in normal cells. (A) Normal prostate cells or DU145 cancer cells were injected with GFP-tagged LC3 plus FADD-DD or treated with TRAIL and followed by fluorescence microscopy. GFP-LC3 forms aggregates (arrows) in FADD-DD-expressing or TRAIL-treated normal cells but does not aggregate in cancer cells. (B) Normal prostate cell survival 24 h after injection with FADD-DD and treatment with zVAD.fmk or 3-MA alone or in combination. FADD-DD-induced cell death is not prevented by either inhibitor alone but is inhibited by the combined inhibitors.

Another characteristic of autophagy is the translocation of LC3 to autophagic vesicles, which can be detected as aggregates of GFP-tagged LC3 (Kabeya *et al.*, 2000). We therefore injected normal primary epithelial cells or cancer cells with untagged FADD-DD or V108E expression vectors along with a GFP-tagged LC3 protein. The aggregation of GFP-LC3 into dots was assessed by fluorescence microscopy (Figure 4A). Aggregation of LC3 occurred in response to FADD-DD in normal cells but not in cancer cells, aggregation started before any morphological signs of cell death

were apparent, and aggregation was not affected by the caspase inhibitor zVAD.fmk (our unpublished data). TRAIL treatment of the cells in the presence of zVAD.fmk had a similar effect. To test whether autophagy contributes to the FADD-DD and TRAIL-induced death that occurs in normal cells, we asked whether the autophagy inhibitor 3-MA could block cell death either on its own or in combination with zVAD.fmk. 3-MA was unable to prevent FADD-DD-induced cell death on its own but did prevent cell death when combined with zVAD.fmk (Figure 4B). These data indicate that autophagy is involved in the caspase-independent cell death response to the FADD-DD signaling pathway in normal epithelial cells.

Selective Disruption of FADD Death Domain-induced Cell Death Occurs When Epithelial Cells Are Immortalized

A distinctive feature of the FADD-DD cell death pathway is that it works in normal prostate epithelial cells but does not work in cancer cells. This raises the question of whether other epithelial cell types behave similarly and, more importantly, when during the transformation process resistance to this pathway arises. To address these questions, we examined human breast epithelial cells that were immortalized and transformed by defined genetic changes (expression of the telomerase catalytic subunit (TERT), SV40 Large T and small t antigens, and oncogenic Ras) (Elenbaas *et al.*, 2001). The cells were derived by expressing the transforming proteins in normal primary human mammary epithelial cells (HMECs) and thus represent a set of cells at different steps in the transformation process arising through defined genetic changes. FADD-DD was expressed in each set of cells by microinjection, and cell death was determined by following the fate of each FADD-DD-expressing cell. Figure 5A shows that normal HMECs and the TERT-expressing HME cells were sensitive to FADD-DD-induced cell death; however, HMECs expressing TERT plus SV40 Large T antigen (HMLcE), Large T and small t antigens (HML), TERT, Large T and small t (HMLE), and cells expressing TERT, Large T, small t, and active Ras (HMLPR) were all resistant to FADD-DD-induced death.

We next asked whether this resistance to cell death was specific to the FADD-DD-induced pathway by comparing the ability of FADD-DD, which cannot activate the caspase-8 pathway, and a full-length FADD protein that can bind caspase-8, to kill HME and HMLcE cells. A general apoptosis resistance mechanism arising in the immortal HMLcE cells should inhibit both FADD proteins. In contrast, a mechanism that selectively disrupts the FADD-DD pathway in HMLcE cells should not alter cell death in response to the FADD molecule that can activate caspase-8. HMLcE cells were resistant to FADD-DD, whereas both HME and HMLcE cells were killed equally well by full-length FADD (Figure 5B). These data indicate that selective resistance to FADD-DD-induced killing arises at a specific step during transformation and can be conferred by a viral oncogene (SV40 Large T antigen). These data also show that the FADD-DD pathway is not affected by TERT expression.

The TERT and T antigen-expressing HMECs are immortal but not transformed (Elenbaas *et al.*, 2001), suggesting that resistance to FADD-DD-induced cell death is associated with immortalization rather than transformation. We therefore tested whether spontaneously immortalized epithelial cells are resistant to FADD-DD. Because human cells very rarely undergo spontaneous immortalization, we used mouse epithelial cells and compared the response to FADD-DD in primary low passage cells to cells that had undergone spontaneous immortalization after continued culture. We also compared the re-

Figure 5. FADD-DD-induced cell death is selectively inhibited in immortalized cells. (A) HMECs at different stages of immortalization and transformation were injected with YFP control or YFP-FADD-DD expression vectors, and the percentage of survival for fluorescent cells was determined. FADD-DD killed normal HMECs and TERT-expressing HME cells, but it did not kill HMEC expressing T antigen plus TERT (HMLcE) or any of the other cells. Panel B, HME cells and HMLcE cells were injected with control, FADD-DD, or a full-length FADD construct that can activate caspase-8. Both FADD-DD and FADD could kill HME cells, but only the FADD molecule capable of activating caspase-8 killed HMLcE cells. These data show that resistance to FADD-DD-induced cell death arises in response to expression of T antigen, which causes immortalization and that this resistance is specific to the FADD-DD pathway.

sponse in primary fibroblasts from the same tissue pieces to test whether the response was epithelial specific. Figure 6A shows that primary mouse mammary epithelial cells (MMECs) were killed by FADD-DD, whereas spontaneously immortalized epithelial cells and primary nonimmortalized fibroblasts were resistant. All the cells underwent apoptosis in response to the full-length FADD protein that can activate caspase-8, indicating that the immortalized cells acquire selective resistance to the FADD-DD pathway rather than a general resistance to all apoptotic stimuli. Similar results were obtained in mouse prostate epithelial cells (our unpublished data). Together with our previous studies (Morgan *et al.*, 2001; Thorburn *et al.*, 2003), these data indicate that human and mouse prostate and breast epithelial cells respond to FADD-DD in the same way.

There are differences in the requirements for immortalization between cell types and between mouse and human cells (Romanov *et al.*, 2001; Drayton and Peters, 2002; Rangarajan and Weinberg, 2003). However, in all cells, it is thought that disruption of p53, INK4a/ARF (these two gene products from the same locus regulate the Rb and p53 pathways), and Rb are important steps in the immortalization process (Drayton and Peters, 2002; Hahn and Weinberg, 2002; Rangarajan and Weinberg, 2003). Tag inactivates p53 and Rb (Ali and DeCaprio, 2001). Disruption of the p53 pathway might therefore provide a simple explanation for the inability of FADD-DD to kill immortal tumor cells. We therefore examined the response to FADD-DD and a FADD molecule that can activate caspase-8 in MMECs from p53 knockout animals. Figure 6B shows that low passage primary epithelial cells from the p53 knockout animals were killed in response to FADD-DD, indicating that loss of p53 function does not affect the FADD-DD pathway and excluding this explanation for the immortalization-dependent resistance. As expected the p53^{-/-} cells did not become senescent and grew well in culture. However, upon continued culture, the cells became resistant to FADD-DD-induced apoptosis but were equally sensitive to apoptosis induced by a FADD molecule that can bind and activate caspase-8. We next cultured epithelial cells from mice with knockouts of the p53 target gene

p21, which controls cell cycle progression, and INK4a/ARF (both genes are inactivated in these animals, which have a deletion of exons 2 and 3; Serrano *et al.*, 1996). In each case, primary MMECs underwent FADD-DD-dependent apoptosis (Figure 6C). As with the p53^{-/-} cells, MMECs that lack functional INK4A/ARF genes became resistant to FADD-DD-induced apoptosis when they were continuously cultured (Figure 5D), suggesting that acquisition of selective resistance to this pathway confers an advantage to the cells.

SV40 T antigen also inactivates Rb, and we next asked whether this was responsible for resistance to FADD-DD. Because Rb knockout results in embryonic lethality (Jacks *et al.*, 1992), we isolated MMECs from animals with homozygous "floxed" Rb genes. These cells were infected with an adenovirus that expresses Cre recombinase to knockout the Rb gene. Three days after infection, there was no detectable Rb protein in the cells (Figure 6C, inset). FADD-DD injection into Rb-deficient cells resulted in apoptosis induction that was equally efficient as that observed with the FADD molecule that can activate caspase-8 (Figure 6C). Together, these data indicate that the FADD-DD pathway is selectively disrupted upon immortalization but that resistance does not arise as a result of the inactivation of p53, INK4a, ARF, p21, or Rb that occurs during immortalization.

Autophagy Is Inactivated in FADD-DD-resistant Epithelial Cells

If autophagy is involved in the FADD-DD pathway, it should occur in early passage mouse breast cells that express FADD-DD and should not be inhibited by zVAD.fmk. However, when cells acquire resistance to FADD-DD-induced cell death, they should also fail to show signs of autophagy. We therefore assessed autophagic vesicle formation in response to FADD-DD in low (passage 4, i.e., sensitive to FADD-DD-induced cell death) and high (passage 30, i.e., insensitive to FADD-DD-induced death) passage MMECs from INK4a/ARF knockout animals. These cells were chosen because they come from the same primary cell prepara-

Figure 6. Inhibition of the FADD-DD pathway in immortal cells is not caused by inactivation of genes that are known to regulate immortalization. (A) Primary mouse mammary epithelial cells, spontaneously immortalized epithelial cells, or primary breast fibroblasts were injected with YFP control, FADD-DD, or FADD expression constructs, and cell survival was determined. All the cell types were killed by the FADD molecule that can activate caspase-8, but only the primary epithelial cells were killed by FADD-DD. (B) Mammary epithelial cells were isolated from p53 knockout mice and tested for sensitivity to FADD-DD after limited culture (passage 2–10) or extended in vitro culture (passage 18–28). FADD-DD killed the low passage number cells but could not kill the high passage number cells. The Western blot insert compares protein samples from the p53 knockout or wild-type animals showing that the cells lacked p53. (C) Primary MMECs were cultured from p21 and INK4a/ARF knockout animals or from animals with floxed Rb genes, which were subsequently infected with a Cre recombinase adenovirus and maintained for 3 d in culture at which time no detectable Rb protein was present (inset). All the low passage (< passage 10) primary cells underwent apoptosis in response to FADD-DD. (D) Low passage (passage 3–8) or high passage (passage 23–27) MMECs from INK4a/ARF $-/-$ mice were injected with FADD-DD as indicated. Only the low passage cells were killed by FADD-DD.

tion and both grow well in culture yet they differ in their ability to die in response to FADD-DD expression. In addition, because the cells lack INK4a and ARF, both the Rb and p53 pathways are inactivated, thus removing potential confounding influences of other cell death pathways. The cells were infected with the FADD-DD- or V108E FADD-DD-expressing adenoviruses, treated with zVAD.fmk to block caspase activation, and assessed by TEM. Low passage cells expressing FADD-DD displayed high levels of autophagic vesicle formation; however, vesicles were not formed in low passage cells expressing the V108E mutant or in high passage cells expressing either wild-type or mutant FADD-DD (Figure 7).

DISCUSSION

In this article, we present data showing that the death domain of FADD can activate a cell death pathway involving both apoptosis and autophagy that is selectively inactivated when normal epithelial cells are immortalized. The same pathway can be activated by TRAIL receptor stimulation and blocked by a FADD-DD mutant, indicating that although the pathway is conveniently activated by expression of exogenous FADD-DD, a physiological signal also can activate this pathway through the endogenous FADD protein. Prostate and breast epithelial cells behave similarly but fibroblasts are unable to activate this pathway. Resistance to

Figure 7. FADD-DD-induced autophagy is lost in late passage epithelial cells. (A) Low or high passage MMECs from INK4a/ARF knockout animals were infected with FADD-DD or V108E FADD-DD adenoviruses in the presence of zVAD.fmk to inhibit caspase-dependent effects and analyzed by TEM for signs of autophagy. Large numbers of vesicles (arrows) were observed only in the low passage cells expressing wild-type FADD-DD. (B) Cell area taken up by vacuolated structures, indicating that FADD-DD but not the V108E mutant causes an increase in such structures in low passage cells.

this form of cell death occurs without affecting other apoptosis pathways, including those that are induced by an FADD protein that can interact with and activate caspase-8 through a different part of the protein. This resistance arises at immortalization rather than complete transformation, suggesting that it represents an early cell death defect that occurs during the development of epithelial cancers. This work provides the first example that we are aware of where apoptosis and autophagy are induced in a cell type-specific manner and selectively disrupted during immortalization and transformation.

Our experiments demonstrating inhibition of TRAIL-induced death by the V108E mutant and cooperation between TRAIL and wild-type FADD-DD to increase killing of normal cells suggest that a stimulus that works through FADD can activate the FADD-DD apoptosis/autophagy pathway. It is important to note that this does not necessarily mean that the normal physiological stimulus is actually TRAIL or that the FADD-DD pathway is an important aspect of TRAIL signaling under normal circumstances. Moreover, because activation of the FADD-DD pathway by TRAIL in normal cells was only detected when we blocked the canonical

caspase-8-dependent pathway, the FADD-DD pathway may not be the major TRAIL-induced cell death pathway even if TRAIL is the physiological stimulus. Instead, it is possible that a different stimulus (perhaps not even involving death receptors) activates FADD to induce the FADD-DD pathway under normal circumstances. Because we have identified a point mutant (V108E) that cannot activate the FADD-DD pathway, it may be feasible to address these issues by creating a knockin mouse that contains the V108E mutation (which, if our ideas are correct, may have a cancer-related phenotype) and asking whether TRAIL signaling occurs properly in these animals.

We have not detected FADD cleavage in normal cells after treatment with TRAIL. In addition, we previously found that overexpression of a full-length FADD molecule containing a point mutation in the DED that prevents caspase-8 binding or expression of wild-type FADD in the presence of a caspase-8 inhibitor could kill normal but not cancerous cells (Thorburn *et al.*, 2003). We therefore do not suggest that the isolated FADD-DD protein occurs under physiological conditions or that the FADD-DD pathway is activated only by the truncated protein. Instead, we think that the FADD-DD pathway is activated by full-length FADD but that this is only evident when the canonical caspase-8 pathway is blocked. We therefore view the expression of the truncated FADD-DD protein, which provides the most effective way to activate this pathway without activating the canonical caspase-8 pathway, as a useful tool to selectively activate and study the pathway that is normally activated by the endogenous full-length FADD protein.

There are other recent examples where autophagy and apoptosis is combined. TRAIL-induced autophagy occurs during breast epithelial cell death to form acini in three-dimensional cultures (Mills *et al.*, 2004). However, this cell death, which occurred in immortal MCF10A cells, was blocked by FADD-DD, suggesting that it has some differences from the FADD-DD-induced death in nonimmortalized cells. In addition, DAP kinase, which has been implicated in death receptor-induced cell death (Cohen *et al.*, 1999), can cause autophagy in addition to apoptosis (Inbal *et al.*, 2002). Beclin 1, which promotes autophagy, is a haplo-insufficient tumor suppressor (Qu *et al.*, 2003; Yue *et al.*, 2003) that displays reduced expression in breast tumors (Liang *et al.*, 1999), providing a genetic link between defects in autophagy and cancer development. Our work suggests that at least some such defects arise at the earliest steps in epithelial cancer development (i.e., the acquisition of immortalization) to inactivate specific cell death pathways that involve both caspase-dependent apoptosis and autophagy.

Although there are differences in the requirements for immortalization and transformation of human and mouse cells (Drayton and Peters, 2002; Rangarajan and Weinberg, 2003), mammary epithelial cells from both organisms behave identically in regards to FADD-DD-induced apoptosis/autophagy and are inhibited by immortalization in both cases. Prostate epithelial cells also behave the same way. Although immortalization is associated with acquired resistance to this cell death pathway, the known activities that are involved in mammalian cell immortalization, including telomerase activation, or loss of function of p53, INK4a, ARF, and pRb are not responsible for resistance to this cell death pathway. In addition, MMECs lacking p53, or INK4a and ARF, which do not undergo crisis or become senescent, become selectively resistant to the FADD-DD pathway upon continued culture. These data suggest that the acquisition of resistance to FADD-DD-induced cell death represents an

uncharacterized aspect of immortalization that confers a selective advantage to the cells.

Although evasion of apoptosis is widely regarded as a hallmark of cancer (Hanahan and Weinberg, 2000), the cell death pathways that must be avoided are poorly understood. Because growth-promoting oncogenic events such as Myc expression or Rb inactivation sensitize cells to diverse apoptotic stimuli and function as an intrinsic tumor suppression mechanism (Lowe *et al.*, 2004), cancer cells must overcome this hurdle to remain below their apoptotic threshold. This can be achieved by altering components of the cell death machinery such as p53, ARF, or Bcl-2 family members that control diverse apoptotic pathways (Lowe *et al.*, 2004). The apoptosis/autophagy pathway that is induced by FADD-DD and TRAIL has unusual characteristics (normal epithelial cell specificity, inactivation when cells are immortalized without affecting other cell death pathways, and no inhibition by loss of p53 or ARF or Bcl-2 expression) that are unlike oncogenic sensitization to apoptosis and suggest it represents a specific hurdle that some cells must also overcome if they are to become cancerous. Further understanding of how the FADD-DD pathway works and why it is not able to work in immortal cells should provide new insights into the role of apoptosis and autophagy dysfunction in the development of epithelial cancers.

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