Tumorigenesis and Neoplastic Progression

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Can Induce Apoptosis in Subsets of Premalignant Cells

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During the transformation from a normal to a malignant cell, several mutations are required to bypass the pathways responsible for controlling proliferation. Premalignant cells have acquired some, but not all of these mutations and consequently have not yet attained a malignant phenotype characterized by tumor formation *in vivo*. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce apoptosis in malignant cells while sparing normal ones and is currently being considered as adjuvant therapy for various human malignancies. Whether TRAIL is effective in inducing apoptosis in premalignant cells is unclear, however. We studied the effect of TRAIL on two human premalignant cell lines the SV7tert and HA1E cells. Both cell lines had been immortalized by the addition of simian virus 40 large T antigen and the telomerase subunit hTERT, but had not been transformed into malignant cells. TRAIL initiated apoptosis by activating both the mitochondrial-independent and -dependent apoptotic pathways in both cell lines at relatively low doses whereas it had no effect on normal human pulmonary artery smooth muscle cells even at high doses. These results suggest that TRAIL can induce apoptosis in premalignant cells and suggests a novel therapy for the treatment of premalignant lesions *in vivo*. (Am J Pathol 2004, 165:1613–1620)

The transition from a normal to a malignant cell occurs through different stages many of which can be identified histologically. The colorectal tumorigenesis model encompassing a range of lesions including adenomas, carcinoma *in situ*, and invasive carcinoma is a well studied example of the progression from a normal to a malignant phenotype.^{1,2} Similarly, in smokers, lesions ranging from squamous metaplasia to carcinoma *in situ* to frank invasive carcinoma demonstrate the presence of premalignant conditions that can be identified in at-risk patients.^{3–5} Although surgical excision of premalignant lesions is possible in some instances this would only be useful for treating identifiable lesions and would likely be limited in practice by excessive morbidity. Chemotherapy would have the theoretical advantage of treating both seen and unseen lesions, but its effectiveness has not been studied in premalignant lesions and systemic toxicity poses a barrier to widespread use.

The progression from a normal to a tumor cell requires that cells first become immortal.^{6,7} This requires an escape from the replicative senescence intrinsic to mammalian cells. In experimental models^{7,8} immortalization can be achieved by inactivating both the retinoblastoma and p53 pathways followed by the expression of the catalytic subunit of telomerase (human telomerase reverse transcriptase or hTERT), the enzyme that elongates telomeres.⁹ Together these strategies allow cells to bypass replicative senescence and be passaged indefinitely. The process of immortalization can be achieved *in vitro* by the introduction of the simian virus 40 (SV40) large T antigen (which inactivates both the retinoblastoma and p53 pathways) and the ectopic expression of

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hTERT, which usually has low or no expression in human somatic cells.^{7,8,10} Transformation into tumor cells (that is the ability of cells to form tumors in immunodeficient mice *in vivo*) requires at least two additional steps: the introduction of oncogenic (constitutively active) *Ras* and the ability to inhibit protein phosphatase 2A. In experimental models these five mutations can transform a normal human epithelial cell into a malignant one.^{7,10}

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family that includes Fas ligand and tumor necrosis factor. Like tumor necrosis factor and Fas ligand, TRAIL induces apoptosis in a variety of tumor cells through binding to its two death receptors DR4 and DR5.^{11,12} Death receptors are characterized by the presence of a cytoplasmic death domain that can bind adaptor proteins (such as Fas-associated with death domain or FADD) to cleave procaspase 8 to its active form. This initiates programmed cell death through two distinct caspase 8-mediated signaling pathways: mitochondrial-independent activation of caspase 3 and mitochondrial-dependent apoptosis because of the cleavage of BID (Bcl-2 inhibitory BH3 domain-containing protein).¹¹ Severe toxicity associated with systemic administration of tumor necrosis factor- α and Fas ligand has limited their use in cancer treatment.^{11,13,14} In contrast, TRAIL can selectively induce apoptosis in malignant cells while sparing normal ones *in vitro*¹² and had no toxic effects when administered systemically in mice and monkeys.¹¹ It is unclear whether TRAIL is effective in inducing apoptosis in premalignant cells, however.

We studied the effect of TRAIL on a cell line derived from a human angiomyolipoma—a nonmalignant tumor that can cause renal failure through local invasion and hemorrhage. This cell line known as the SV7tert was immortalized by the addition of the SV40 large T antigen and hTERT.¹⁵ We also studied a cell line derived from a normal human renal epithelial cell (HA1E) that was also immortalized by the addition of SV40 large and small T antigen and hTERT.¹⁶ Neither of these cell lines was transformed and therefore are considered premalignant cells. TRAIL rapidly induced apoptosis in these cells at relatively low doses whereas it had no effect on normal human pulmonary artery smooth muscle cells (PA SMCs) even at very high doses. TRAIL initiated apoptosis by activating both the mitochondrial-independent and -dependent apoptotic pathways. Inhibiting new protein synthesis by the addition of cycloheximide increased apoptosis in TRAIL-treated cells. These results suggest that TRAIL can induce apoptosis in premalignant cells and raise the possibility that it may have chemopreventive activity against premalignant lesions such as squamous metaplasia and carcinoma *in situ* that are not amenable to surgical therapy.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium was obtained from CellGro (Mediatech, Herndon, VA). Trypsin-ethylenedia-

minetetraacetic acid, L-glutamine from Life Technologies, Inc. (Grand Island, NY), fetal bovine serum from Gemini (Woodland, CA), polyvinylidene difluoride membrane, ECL-plus from Amersham (Buckinghamshire, UK). Cycloheximide (Sigma, St. Louis, MO) was dissolved in ethanol. TRAIL was obtained from R&D Systems (Minneapolis, MN) and dissolved in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin. Mouse anti-human caspase 8 monoclonal antibody (mAb), rabbit polyclonal anti-PARP, and rabbit polyclonal anti-Bak were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal rabbit anti-BID, polyclonal anti-human cleaved caspase 3, polyclonal rabbit anti-Smac/DIABLO, and anti-human cleaved caspase 9 were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-cFLIP was obtained from Affinity Bioreagents Inc. (Golden, CO). Mouse anti-human Bcl-X_L (mAb) and mouse anti-Bax (mAb) were obtained from BD Pharmingen (San Diego, CA). Mouse anti-human β -actin was obtained from Sigma. Secondary horseradish peroxidase-conjugated antibodies: donkey anti-mouse and -rabbit were obtained from Jackson Labs (West Grove, PA).

Cell Culture and TRAIL Treatment

Human PA SMCs were obtained from Clonetics (Walkersville, MD) and grown in SMGM2 media that contains 5% fetal bovine serum. SV7tert (American Type Culture Collection, Rockville, MD) is a cell line derived from a human angiomyolipoma through the sequential introduction of SV40 large T antigen and hTERT into primary human angiomyolipoma cells¹⁵ and grown in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/L L-glutamine, and 10% fetal bovine serum. HA1E is a cell line derived from normal human renal epithelium through the introduction of SV40 large and small T antigen and hTERT.¹⁶ Cells were grown in humidified Forma Scientific incubators (Midland, MA) at 37°C in 5% CO₂, 16% O₂, balance nitrogen. Smooth muscle cell identity was verified for the human PA SMCs by positive staining for smooth muscle α -actin (mouse monoclonal anti-body, Sigma). Cells were seeded at 1×10^6 cells per 60-mm cell culture plate and incubated for 24 hours before recombinant human TRAIL was added. TRAIL was dissolved in sterile PBS plus 0.1% bovine serum albumin and diluted in fresh tissue culture medium before being added to cells.

Flow Cytometry Apoptosis Assay

After a 6-hour incubation with various concentrations of TRAIL cells were trypsin-ethylenediaminetetraacetic acid-digested from culture plates and the trypsin inactivated by addition of Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells were collected by low centrifugation, washed with PBS, and recollected by centrifugation. After the final wash cells were incubated for 15 minutes in annexin V and propidium iodide using the Vybrant apoptosis assay kit (catalog no. 13242; Molecular Probes, Eugene, OR). Cells were analyzed

within 60 minutes at the University of Colorado Cancer Center Flow Cytometry Core.

Determination of Caspase Activation by Flow Cytometry

This method utilizes flow cytometry to quantitate the amount of activated caspase 3, Bcl-2, and cleaved PARP in whole cell lysates. Three bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for cleaved PARP (C-PARP), Bcl-2, and activated caspase 3 (human apoptosis kit, catalog no. 557816; Becton-Dickinson, Mountain View, CA). After a 6-hour incubation with either control or TRAIL at 100 ng/ml, whole cell lysates were harvested as described in the manual and incubated with these beads. The beads were then analyzed in the University of Colorado Cancer Center Flow Cytometry Core. The y axis indicates the distinct bead identification number based on increasing red fluorescence intensity whereas the x axis indicates the fluorescence intensity of the phycoerythrin-conjugated detection reagent.

DNA Fragmentation Assay

After a 24-hour incubation in 100 ng/ml of TRAIL, cells were collected for DNA isolation using TACS DNA laddering kit from R&D Systems. Isolated DNA was separated on a 2% agarose gel stained with ethidium bromide.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Quantitative reverse transcriptase-polymerase chain reaction assays were performed using the ABI 5700 real-time system with SYBR green fluorescence with normalization to β -actin, as previously described.¹⁷ RNA was prepared using an Rneasy kit (Qiagen, Valencia, CA). Primers were designed using the GenBank sequence for the human DR4 death receptor and β -actin (DNA Technologies, Coralville, IA). These included the human DR4 primers: AGCATGT-CAGTGCAAACCCAGG, TCCAGGGCGTACAATCCTTG; human DR5 primers: TGCATCTCCTGCAAATATGGAC, TGCAGGGACTTAGCTCCACTTC; human β -actin primers: GTCTTCCCCTCCATCGTG, GGATGCCTCTCTTGCTCTG.

Western Blot

At the scheduled time points, cells were washed with cold PBS, lysed in radioimmunoprecipitation (RIPA) buffer [PBS, 1% ipegal, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, phenylmethyl sulfonyl fluoride (10 mg/ml), aprotinin (30 μ l/ml), and sodium orthovanadate (1 mmol/L)], pelleted at 14,000 $\times$ g, and the protein concentration of the supernatant determined by Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions (1% β -mercaptoethanol) using 8 to 16% gradient gels. The

proteins were then transferred to polyvinylidene difluoride membrane in 20% methanol. Membrane was blocked in 5% nonfat milk and 0.1% Tween-20, probed with appropriate antibodies (see Materials), detected with appropriate secondary antibodies conjugated to horseradish peroxidase at 1:10,000 dilution and detected using ECL-plus. Protein expression was quantified using NIH image 1.63 and expressed as arbitrary density units relative to control.

Results

TRAIL Selectively Induces Apoptosis in SV7tert Cells

To determine whether TRAIL could induce apoptosis in SV7tert cells (derived from a human angiomyolipoma) we exposed them to increasing concentrations of TRAIL. Human PA SMCs were used as a control. After a 6-hour incubation with TRAIL at 100 ng/ml, apoptotic morphological changes including cell rounding, shrinkage, and detachment could be seen under light microscopy in SV7tert (Figure 1A), but not control cells (data not shown). The human PA SMCs did not exhibit any morphological changes even after 48 hours of treatment (data not shown). Flow cytometry analysis demonstrated apoptosis in TRAIL-treated SV7tert cells after 6 hours at concentrations as low as 10 ng/ml. TRAIL did not cause apoptosis in human PA SMCs even at 800 ng/ml (Figure 1B). Intranucleosomal fragmentation (DNA laddering) 24 hours after treatment with TRAIL confirmed that cell death occurred by apoptosis (Figure 1C). mRNA for the TRAIL R1/DR4 and R2/DR5 receptors was present in both cell types in approximately equivalent amounts as determined by quantitative reverse transcriptase-polymerase chain reaction. This indicated that the inability of TRAIL to induce apoptosis in the control PA SMCs was not because of the lack of TRAIL receptors.

TRAIL Initiates Apoptosis in SV7tert Cells through Activation of Caspase 8

TRAIL initiates apoptosis through binding to its death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5. The trimerization of TRAIL receptors recruits the adaptor protein, FADD, to form the death-inducing signal complex (DISC). This complex recruits procaspase 8 and induces its proteolytic cleavage to the active (cleaved) form thereby initiating the subsequent downstream activation of effector caspases.¹¹ TRAIL can also cause cell necrosis through a caspase 8-independent pathway, however.^{12,18} To determine whether TRAIL activated caspase 8 in the SV7tert cells we added increasing doses of TRAIL and harvested cell lysates for protein analysis. Figure 2A indicates that cleaved (activated) caspase 8 can be detected after stimulation with TRAIL in a dose-dependent manner. A time course indicates that cleaved caspase 8 is detectable within 30 minutes of TRAIL treatment and becomes prominent by 1.5 hours (Figure 2B).

Figure 1. TRAIL induces apoptosis in SV7tert cells but not in human PA SMCs. **A:** Light microscopy of SV7tert cells exposed to TRAIL (0 or 100 ng/ml) for 6 hours. Human PA SMCs showed no changes (not shown). **B:** Effect of increasing doses of TRAIL at 6 hours on apoptosis in human PA SMCs and SV7tert cells as determined by flow cytometry. Data are mean $\pm$ SEM, $n = 3$ separate experiments; *, $P < 0.05$ versus human PA SMCs. **C:** Ethidium bromide-stained agarose gel of DNA harvested from human PA SMCs and SV7tert cells 24 hours after exposure to 100 ng/ml of TRAIL. DNA laddering was seen only in the SV7tert cells.

TRAIL Activates Both the Mitochondrial-Dependent and -Independent Apoptotic Pathways in SV7tert Cells

TRAIL can induce apoptosis in certain cell types (such as B- and T-cell lymphoma cell lines) solely through direct activation of the caspase pathway. In other cells TRAIL also requires activation of the mitochondrial pathway with the release of cytochrome c and Smac/DIABLO (which leads to the activation of caspase 9) to cause apoptosis. We demonstrated above that TRAIL-stimulated SV7tert cells activated caspase 8. Next we wanted to confirm that the principal downstream effector caspase, caspase 3, was also activated. Protein from SV7tert cells was harvested after treatment with TRAIL (100 ng/ml) and cell lysates were analyzed for evidence of active caspases by

Western blotting. Figure 3A indicates that caspase 3 is cleaved and active in TRAIL-treated SV7tert cells. Activated caspase 3 is the main executor of apoptosis in cells and results in the cleavage of multiple cytoplasmic and nuclear proteins. One of its targets is the nuclear enzyme poly (ADP-ribose) polymerase (PARP). Cleavage of PARP confirms complete activation of the apoptotic pathway by TRAIL (Figure 3A).

To demonstrate that TRAIL also activated the mitochondrial-dependent apoptotic pathway we show that the proapoptotic protein, BID, is cleaved by caspase 8 into truncated BID (Figure 3B). Truncated BID binds the mitochondrial membrane causing membrane depolarization and this leads to the release of Smac/DIABLO into the cytosol.¹¹ Figure 3B demonstrates the increase in Smac/DIABLO after TRAIL stimulation and demonstrates

Figure 2. TRAIL activates caspase 8 in angiomyolipoma cells. **A:** Western blot of SV7tert cell lysates harvested 30 minutes after exposure to increasing doses of TRAIL. **B:** Western blot showing the time course of caspase 8 activation in SV7tert cells treated with 100 ng/ml of TRAIL. Representative blot from three separate experiments.

Figure 3. TRAIL activates both the mitochondrial-independent and -dependent apoptotic pathways in angiomyolipoma cells. **A:** Western blot of SV7tert cell lysates harvested 6 hours after exposure to 100 ng/ml of TRAIL shows cleavage (and activation) of caspase 3. Cleavage of the nuclear protein PARP confirmed complete activation of the caspase pathway. **B:** TRAIL cleaves BID to its truncated form indicating activation of the mitochondrial-dependent apoptotic pathway. The presence of Smac/Diablo and cleaved caspase 9 confirm this observation. Representative blot from three separate experiments. Truncated BID shown at 2.5 hours; Smac/Diablo and cleaved caspase 9 are shown at 6 hours.

Figure 4. Inhibiting protein synthesis with cycloheximide (CHX) amplifies TRAIL-induced apoptosis in angiomyolipoma cells. **A:** Effect of TRAIL alone and in combination with CHX (25 μ mol/L) on apoptosis of SV7tert cells as determined by flow cytometry. Data are mean $\pm$ SEM, $n = 3$ separate experiments; *, $P < 0.05$ versus TRAIL (100 ng/ml) alone. **B:** Western blot of SV7tert cell lysates harvested 6 hours after TRAIL exposure demonstrating increased activation of caspases 8 and 9 along with an increase in cleaved PARP in the presence of CHX. Representative blot from three separate experiments.

that caspase 9 is cleaved and activated. These results indicate that TRAIL activates both the mitochondrial-independent and -dependent pathways in SV7tert cells.

Inhibiting Protein Synthesis Significantly Enhances TRAIL-Induced Apoptosis in SV7tert Cells

TRAIL resistance has been demonstrated in some cell lines and the mechanism of resistance can vary depending on cell type.^{19–21} In addition, TRAIL can modulate its own apoptotic effect through the up-regulation of anti-apoptotic proteins. We had already shown that the SV7tert cells were sensitive to TRAIL. Next we wanted to determine whether modulators of apoptosis were induced by TRAIL. Inhibiting new protein synthesis by the addition of cycloheximide significantly increased apoptosis in TRAIL-treated SV7tert cells (Figure 4A). Consistent with this the caspase cascade was activated to a greater degree in the presence of cycloheximide (caspase 8, caspase 9, and PARP as shown in Figure 4B; caspase 3 not shown).

TRAIL Causes Apoptosis in HA1E Cells

To determine whether TRAIL could induce apoptosis in another immortalized, premalignant cell line we examined its effect on HA1E cells. This is a cell line derived

Figure 5. TRAIL induces apoptosis in HA1E cells. Effect of increasing doses of TRAIL at 6 hours on apoptosis in human PA SMCs, SV7tert, and HA1E cells as determined by flow cytometry. Data are mean $\pm$ SEM, $n = 4$ separate experiments; *, $P < 0.05$ for both SV7tert and HA1E cells versus human PA SMCs. These apoptosis experiments were done simultaneously in all three cell lines and represent different experiments from those shown in Figure 1B.

from normal human renal epithelial cells immortalized by the addition of the SV40 large T antigen and hTERT.¹⁶ To first determine whether SV7tert and HA1E cell lines had acquired secondary mutations capable of transforming them into a malignant phenotype, we assessed their ability to grow in soft agar. The ability of cells to grow on soft agar is an indicator of malignant potential although it is less reliable as a marker of malignant transformation than *in vivo* tumorigenesis.²² HA1E cells did not form colonies in soft agar (0.5 colonies at 2 weeks) whereas SV7tert cells were able to form some colonies (120 ± 13 colonies at 2 weeks). In contrast, a transformed endothelial cell line (the SVR line) that has been demonstrated to induce tumors *in vivo*²² generated 2039 colonies in soft agar. We had previously demonstrated that unlike SVR cells, SV7tert cells could not generate tumors when injected into nude mice.¹⁵ Therefore the weak ability of SV7tert (relative to SVR) cells to grow some colonies in soft agar suggests that although they may have more malignant potential than HA1E cells, they have not yet attained a malignant phenotype.

TRAIL induced apoptosis in HA1E cells throughout a similar dose range as that seen in SV7tert cells (Figure 5). To determine whether TRAIL also activated the caspase system we used a new technology that utilizes flow cytometry to assess activation of multiple parts of the apoptotic pathway at one time. We exposed human PA SMCs, SV7tert, and HA1E cells to TRAIL for 6 hours and then harvested protein from cell lysates. Cell lysates were then incubated with beads specific for either activated caspase 3, Bcl-2, or cleaved PARP and analyzed by flow cytometry. Figure 6 demonstrates the type of data produced by this method (lower band is activated caspase 3, middle band is Bcl-2, and the upper band represents cleaved PARP); the graphs on the right demonstrate the statistical analysis of repeated experiments. Activated caspase 3 and cleaved PARP is present in SV7tert and HA1E cells, but not in human PA SMCs exposed to TRAIL. Expression of the anti-apoptotic protein, Bcl-2, does not change in TRAIL-treated cells.

Discussion

Early detection and treatment of malignant lesions is critical to controlling the devastating effects of cancer. The histological and molecular changes associated with the transformation from a normal to a malignant cell are well described in numerous cancers including colon,^{1,2} lung,^{23,24} and cervical.²⁵ Therapy currently consists primarily of surgical or endoscopic excision of offending cells. Unfortunately surgical intervention is effective only on specific, identifiable lesions and even then it carries significant morbidity. In addition, surgery is frequently not a therapeutic option in patients with advanced pulmonary disease who are at highest risk for developing premalignant lesions such as squamous metaplasia and carcinoma *in situ* that can ultimately lead to the development of bronchogenic carcinoma. Clinical investigators using fluorescent (LIFE) bronchoscopes and targeted endobronchial biopsies (performed both before and after chemotherapy) have noticed a decrease in premalignant lesions (squamous metaplasia, carcinoma *in situ*) in patients after chemotherapy for surgically unresectable lung cancer (B. Keith, Y. Miller, personal communication). This suggests that chemotherapy might be useful in targeting these premalignant lesions if less toxic agents were identified.

In vitro cell models designed to study the progression from normal to malignant cells have been described.^{8,10,26,27} The transition from a normal to a malignant phenotype *in vitro* first requires the cell to become immortalized. Mutating (bypassing) the retinoblastoma and p53 pathways through the introduction of the SV40 large T antigen coupled with the introduction of the telomerase catalytic subunit hTERT is sufficient to immortalize human epithelial cells and render them resistant to replicative senescence.⁸ These immortalized cells are not considered transformed or malignant however because they are unable to form tumors when injected into immunodeficient mice. The addition of oncogenic (constitutively active) *ras* along with the addition of the SV40 small T antigen (which inactivates protein phosphatase 2A) is required to fully transform cells. Therefore, introducing the SV40 early region elements (containing both the large and small T antigen), hTERT, and oncogenic *ras* is sufficient to completely transform a normal human epithelial cell into a malignant one *in vitro*.^{8,10}

The relevance of this *in vitro* cell model to premalignant and malignant lesions *in vivo* is not clear. The somatic and germline mutations that affect the cell genome and contribute to the development of cancer can range from point mutations to changes in chromosome number; advanced, aggressively growing tumor cells frequently have highly mutated genomes. The common finding of alterations in karyotype and changes in gene number (aneuploidy) in both naturally occurring tumors in humans and experimentally induced ones in animals suggests that the development of aneuploidy with the resulting disruption of a large array of normal genes is required for tumorigenesis. Others have suggested that only specific mutations are required to transform cells and the genetic instability seen in advanced malignancies is nothing more than an effective means of rapidly gener-

Figure 6. TRAIL activates caspase 3 and cleaves PARP in HA1E and SV7tert cells, but not human PA SMCs. Three bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for cleaved PARP (C-PARP), Bcl-2, and activated caspase 3 (human apoptosis kit from Becton-Dickinson, see Materials and Methods). Six hours after exposure to control or TRAIL (100 ng/ml) whole cell lysate protein from human PA SMCs (**A**), HA1E cells (**B**), and SV7tert cells (**C**) was harvested, normalized, and then incubated with these beads. The beads were then analyzed by flow cytometry. The y axis indicates the distinct bead identification number based on increasing red fluorescence intensity (bead ID FI). The **top band** (most intense) represents C-PARP, the **middle band** represents Bcl-2, and the **bottom band** (least intense) represents activated caspase 3. The x axis indicates the fluorescence intensity of the phycoerythrin-conjugated detection reagent (FI of PE). The figures on the **left** demonstrate data obtained from a single experiment; the bar graphs on the **right** indicate the statistical analysis of four separate experiments. *, *P* value < 0.05. Each experiment was compared to its (untreated) control and changes denoted by fold-change over baseline. Note that after incubation with TRAIL the fluorescence intensity of activated caspase 3 and cleaved PARP increases (ie, shifts to the right) in HA1E and SV7tert cells, but not in human PA SMCs. Bcl-2 is unchanged in any of the three cell types after TRAIL exposure.

ating the required number of mutant alleles required for neoplastic growth. In this case selectively mutating the required pathways should be sufficient to transform a normal cell into a malignant cell even in the absence of the genetic instability that leads to aneuploidy. Zimonjic and colleagues¹⁰ have demonstrated this to be true. By the introduction of the SV40 early region elements together with a *ras* oncogene and hTERT they were able to generate tumorigenic cells without widespread genetic instability.

These cell models with well-defined mutations, but absent the genetic chaos commonly seen in naturally or experimentally induced tumors, can provide information about cell behavior at different points along the road to malignancy; more importantly they can provide information about cell susceptibility to chemotherapeutic agents

at these different stages. The minimum number of mutations required to induce TRAIL sensitivity is not known. Using this model we tested whether premalignant cells that had been immortalized, but not yet transformed, were sensitive to TRAIL. We demonstrated that TRAIL could induce apoptosis in both SV7tert and HA1E cells. TRAIL activated both the mitochondrial-dependent and mitochondrial-independent apoptotic pathways in these cells establishing that premalignant cells are sensitive to TRAIL. In contrast normal human PA SMCs that had not been immortalized remained resistant to TRAIL even at high doses for prolonged duration. No toxic effects of TRAIL were seen in the normal (nonimmortalized) human PA SMCs.

The ability of SV7tert cells to form some colonies in soft agar may indicate that they have a greater malignant

potential or are closer to a malignant phenotype than the HA1E cells. Another possibility is that the different origins of the cell lines (a normal renal epithelial cell in the case of HA1E compared to a human renal angiomyolipoma in the case of SV7tert cells) may be responsible for this growth difference. The hallmark of malignant transformation, however, is the ability to generate tumors when injected *in vivo* and SV7tert cells are unable to do this.¹⁵ In contrast, a truly transformed cell line such as the SVR cell that can form tumors *in vivo*²², developed in excess of 2000 colonies in soft agar (compared to 120 for the SV7tert cells) under similar conditions. We interpret these results to indicate that SV7tert cells still represent a pre-malignant phenotype despite a more aggressive growth pattern than HA1E cells.

These results demonstrate that the initial step toward malignant transformation, the immortalization of a cell, renders it sensitive to a chemotherapeutic agent, TRAIL, which has no effect on normal cells. Whether this observation is applicable to all premalignant cells is uncertain because we examined only two cell lines both of renal origin. Additional studies to determine TRAIL's effectiveness in other premalignant cells will be needed. These results do raise the possibility, however, that TRAIL might be effective in treating premalignant lesions *in vivo* with minimal toxic effects.

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