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Award Number: DAMD17-01-1-0212

TITLE: The Paracrine Induction of TRAIL by Genotoxic Agents

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REPORT DATE: June 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188		
Public reporting burden for this collection of infor the data needed, and completing and reviewing the reducing this burden to Washington Headquarter Manacement and Budget. Paperwork Reduction	mation is estimated to average 1 hour per response his collection of information. Send comments regar s Services, Directorate for Information Operations a Project (0704-0188). Washington, DC 20503	, including the time for reviewing instruction ding this burden estimate or any other a nd Reports, 1215 Jefferson Davis High	ctions, searching exis aspect of this collecti way, Suite 1204, Arti	sting data sources, gathering and maintaining ion of information, including suggestions for ington, VA 22202-4302, and to the Office of	
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### Summary

TNF related apoptosis inducing ligand, TRAIL, is a recently cloned cytokine that has been shown to induce apoptosis in a synergistic fashion with chemotherapeutic agents on several cancer cell lines. Xenografts of several carcinoma cell lines demonstrate TRAIL and chemotherapy to cause complete regression of established tumors. Etoposide, a topoisomerase type II inhibitor, induces the upregulation of TRAIL in several breast and lung carcinoma lines. Analysis of human lung tumors demonstrates TRAIL mRNA expression is significantly decreased in tumor relative to autologous non-tumor lung tissue. Genotoxin-induced apoptosis of human cancer cells correlates with TRAIL surface expression. Expression of TRAIL and its death receptor, DR5, is regulated by NFKB. NFKB inhibition results in aggressive growth and chemotherapy resistance of H157 human lung squamous carcinoma. Gene profiling of H157 cells demonstrates that NFKB regulates the expression of both pro- and anti-apoptotic proteins including inhibitors of apoptosis (IAPs) in addition to DR5 and TRAIL. It is the predominance of the opposing NFKB-dependent signals that dictates the cell's decision to survive or die. Loss of TRAIL expression in human lung cancer provides a means by which tumors can avert programmed cell death. DcR1, a GPI-linked TRAIL receptor, has been proposed to confer TRAIL resistance by serving as a membrane bound TRAIL trap. DcR1 expression inhibits caspase 8 and BID cleavage as well as JNK phosphorylation induced by etoposide and TRAIL. DcR1, however, also induces potent survival signals. Restoration of DcR1 in breast carcinoma cells to physiologic levels induces Akt activation in a src family kinase dependent manner. DcR1 expression confers resistance to mitochondria insult and apoptosis induced by currently utilized chemotherapeutic agents as well as to Antimycin A, a BH3 domain homologue that targets mitochondria directly. These data indicate that in addition to inhibiting DR4 and DR5 signaling by binding TRAIL, DcR1 also influences cell survival by preserving mitochondrial integrity. DcR1 is a regulator of the apoptosis module whose expression enhances cell survival and confers resistance to chemotherapy. This work demonstrates the importance of TRAIL and its receptors in neoplasia response to DNA damaging agents.

### Results

## Expression of the TRAIL system is altered in lung cancer.

Since genotoxins are known to activate the apoptotic module through death receptor ligands, we assessed the significance of the TRAIL/death receptor pathway in human lung cancers compared to autologous non-tumor lung tissue. This analysis gives a direct comparison between tumor and non-tumor lung tissue from the same patient, an analysis rarely available for most lung tumor samples. Seven human adenocarcinoma and two squamous carcinomas were compared to autologous lung tissue for the expression of TRAIL mRNA and its receptors DR4,

DR5 and DcR1. No dramatic changes were detected for expression of DR4, DR5 or DcR1 in any of the tumors compared to the autologous lung. In contrast, in seven of nine tumors the mRNA expression for TRAIL was significantly reduced (p<0.003). Lung tumors demonstrate a loss of TRAIL expression compared with adjacent non-tumor autologous lung, suggesting a loss of death receptor mediated apoptotic activity in lung malignancy. Although the function of TRAIL in normal lung is unknown, decreased TRAIL expression in tumors may confer chemotherapy resistance to particular genotoxins and provide a potential survival advantage for tumor cells.

# Suppression of death receptor signaling leads to chemo-resistance.

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To test the hypothesis that loss of TRAIL/death receptor activity may lead to chemoresistance, we assessed the correlation of TRAIL expression with apoptotic response in epithelial cell lines. There is a strong correlation between the induction of cell surface TRAIL expression and the sensitivity of epithelial cells to undergo apoptosis in response to a genotoxic stimulus. Cell surface TRAIL expression correlates with the apoptotic response to etoposide for primary human airway and breast epithelium, H157 lung squamous carcinoma cells, A549 lung adenocarcinoma cells, ZR-75-1, and MDA231 human breast adenocarcinoma cells. To test the prediction that cell surface TRAIL expression is an important autocrine or paracrine response to genotoxin-induced cell death, the GPI-linked decoy receptor, DcR1, which binds TRAIL but does not signal caspase activation was expressed in H157 cells. DcR1 functions to bind TRAIL and effectively block TRAIL-induced apoptosis.

The etoposide dose response curves demonstrated that as the dose of genotoxin increases, the degree of apoptosis also increases in WT H157 cells. With etoposide doses ranging from  $1\mu$ M to  $100\mu$ M, WT H157 cells displayed apoptotic responses ranging from 18-52% with 48-hr treatment. H157 cells, which stably express DcR1 as confirmed by flow cytometry surface expression, suppress etoposide-induced apoptosis. H157 cells stably expressing DcR1 suppress etoposide induced apoptosis rates by greater than 50% to levels of 6-24% over the same dose range. DcR1 expression has a strong protective effect against etoposide induced cell death, consistent with a significant role for TRAIL in the death response and chemo-resistance to etoposide. A similar effect of DcR1 is seen in MDA 231 cells, demonstrating the protective role of DcR1 is a general function of the decoy receptor and not a unique response to H157 cells. The protective effect of DcR1 expression is selective for genotoxins such as etoposide and doxorubicin and is not seen with paclitaxel-induced H157 cell death, demonstrating a role for TRAIL expression in the apoptotic response to specific genotoxins.

# Loss of NFKB-dependent TRAIL expression leads to epithelial cell chemo-resistance and aggressive tumor growth.

NF $\kappa$ B activation has been shown to be required for the expression of TRAIL and its death receptors. To define NF $\kappa$ B function in response to genotoxins, dominant negative I $\kappa$ B $\alpha$  (DNI $\kappa$ B $\alpha$ ) was stably expressed in H157 cells. This mutant I $\kappa$ B $\alpha$  contains serine to alanine conversions at residues 32 and 36 that abolish the ability of IKK $\alpha$  to phosphorylate these sites. This prevents ubiquitination and degradation of mutant I $\kappa$ B $\alpha$  and subsequent dissociation and nuclear translocation of NF $\kappa$ B.

H157 cells stably expressing DNIkBa were screened for loss of NFkB DNA binding activity following phorbol ester (PMA) treatment. H157 cells expressing DNIkBa lose PMA-

stimulated NFkB DNA binding activity. Inhibition of NFkB DNA binding activity in response to PMA is specific, as AP-1 DNA binding activity remains intact in the same nuclear extracts.

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Loss of NF $\kappa$ B DNA binding activity was also examined in DNI $\kappa$ B $\alpha$  H157 cells treated with etoposide. Both wild type (WT) H157 cells and DNI $\kappa$ B $\alpha$  H157 cells were treated with etoposide for 12 and 24 hours, and nuclear extracts were harvested and subjected to EMSA. NF $\kappa$ B DNA binding activity was induced following treatment of H157 cells for 12 and 24 hours with etoposide, while DNI $\kappa$ B $\alpha$  H157 nuclear extracts show loss of NF $\kappa$ B DNA binding activity. Etoposide treatment of WT H157 cells results in decreased levels of I $\kappa$ B $\alpha$  protein detected by immunoblotting, reflective of the phosphorylation, ubiquitination, and protein degradation of I $\kappa$ B that allows nuclear translocation of NF $\kappa$ B. DNI $\kappa$ B $\alpha$  H157 cells retain stable and consistent cytosolic levels of I $\kappa$ B protein despite treatment with etoposide. These studies confirm that DNI $\kappa$ B $\alpha$  effectively abolishes the nuclear translocation of NF $\kappa$ B, preventing transcriptional activation of NF $\kappa$ B target genes.

To define the importance of NFkB in etoposide-induced apoptosis, WT H157 cells and DNIkB $\alpha$  H157 cells were treated with etoposide, and apoptosis was quantitated by acridine orange. Basal levels of apoptosis in unstimulated WT H157 cells was approximately 5%±1.6% and increased to 31%±2.1% and 42.4%±4.7% with 24 hour etoposide exposure at 30µM and 100µM, respectively. Apoptosis in unstimulated DNIkB $\alpha$  H157 cells was comparable to WT H157 at 6%±2%. With 30µM etoposide, the apoptotic response was suppressed 50% or greater in DNIkB $\alpha$  relative to WT H157 cells (from 31%±2.1% in WT H157 to 16.9%±6.3% for DNIkB $\alpha$  H157 cells seen in . Similarly, the 42.4%±4.7% apoptotic cells observed with 100µM etoposide treatment for 24 hours in WT H157 cells decreased to 20.2%±4.2% in DNIkB $\alpha$  H157 cells. These studies demonstrated that NFkB activity is required for maximal apoptotic response to etoposide insult in H157 carcinoma cells. Similar results were obtained with other human carcinoma cell lines including ZR-75-1 breast adenocarcinoma cells.

To assess further the biologic implications of NFkB inhibition, WT and DNIkB $\alpha$  H157 cells were examined for tumorigenic potential in nude mouse xenografts.  $3x10^{6}$  WT H157 or DNIkB $\alpha$  H157 cells were injected into nude mice, and tumor volumes were followed for 28 days at which time tumor volumes necessitated animal sacrifice. WT and DNIkB $\alpha$  H157 mouse xenografts grew comparably until day 15, when separation of tumor growth curves becomes apparent. DNIkB $\alpha$  H157 xenografts grow to a larger size over a shorter time frame compared with WT H157 xenografts with growth differences reaching statistical significance by day 17. The aggressive tumor growth characteristic of DNIkB $\alpha$  H157 xenografts is not the result of a shortened cell cycle compared with WT H157 cells. In fact, *in vitro* growth curves suggest that DNIkB $\alpha$  H157 cells grow with a slower doubling time compared to WT H157 cells.

## NFKB is required for genotoxin but not paclitaxel-induced apoptosis.

Overexpression of DcR1 inhibits etoposide and doxorubicin induced apoptosis, while paclitaxel-induced apoptosis is unaffected by DcR1 levels. To determine the specificity of NF $\kappa$ B dependent genotoxin induced apoptosis, WT H157 and DNI $\kappa$ B $\alpha$  cells were subjected to treatment with doxorubicin in comparison with etoposide. Treatment of WT H157 cells with 300nM doxorubicin results in 32.0% apoptosis over 48 hours. This is inhibited by almost 50% in DNI $\kappa$ B $\alpha$  H157 with an apoptotic index of 17.2%. This is comparable apoptotic suppression as seen with 100 $\mu$ M etoposide treatment. In comparison, shows the response of WT and DNI $\kappa$ B $\alpha$  H157 cells exposed to 30nM and 300nM paclitaxel for 24 hours. Fifty to 52% of WT H157 cells are apoptotic after 24 hours for both doses and 39% and 46% of DNI $\kappa$ B $\alpha$  H157 cells were apoptotic after 30nM and 300nM paclitaxel treatment, respectively. While paclitaxel-induced apoptosis was independent of NF $\kappa$ B activation, loss of NF $\kappa$ B expression in DNI $\kappa$ B $\alpha$  results in chemo-resistance to etoposide and doxorubicin.

To validate the etoposide chemo-resistance of DNI $\kappa$ B $\alpha$  cells, athymic nude mice harboring WT H157 or DNI $\kappa$ B $\alpha$  xenografts were treated with weekly doses of etoposide or paclitaxel beginning 7 days post-xenograft injection. WT H157 xenograft growth is suppressed with etoposide treatment with a separation of growth curves reaching statistical significance by day 21.

In contrast, DNI $\kappa$ B $\alpha$  xenografts are chemo-resistant to etoposide. Despite treatment with etoposide, DNI $\kappa$ B $\alpha$  xenografts continue to grow at the same rate as untreated DNI $\kappa$ B $\alpha$ . Paclitaxel treatment of mice with DNI $\kappa$ B $\alpha$  xenografts results in suppression of tumor growth comparable to WT H157 cells. These findings indicate that inhibition of NF $\kappa$ B function in H157 cells results in a more aggressive tumor that is refractory to treatment with etoposide, similar to the anti-apoptosis and chemo-resistance of DNI $\kappa$ B $\alpha$  seen in H157 cells assayed *in vitro*.

### Etoposide induces surface expression of DR5 and its ligand, TRAIL.

To assess the surface expression of TRAIL and DR4/DR5 for WT and DNIkBa H157 cells in response to etoposide, cells were treated with 100µM etoposide for 24 hours and subjected to flow cytometric analysis with specific antibodies to DR4, DR5 and TRAIL. The surface expression of DR4 in WT H157 and DNIkBa H157 cells with and without etoposide treatment was measured. WT H157 cells demonstrate a DR4 surface expression at a ratio of 15.1 and 18.8 relative to background with and without etoposide treatment, respectively. No significant change in DR4 surface expression is seen in DNIkBa H157 cells, which demonstrate comparable DR4 surface expression ratios of 11.2 and 17.3 with and without etoposide treatment, respectively. The lack of changes in DR4 expression correlates with RNase protection assay for DR4 transcript, which were unchanged with etoposide treatment in WT and DNI $\kappa$ B $\alpha$ H157 cells. In contrast to DR4, DR5 surface expression increases with etoposide treatment in WT H157 cells, and this induction of surface expression is inhibited in DNIkBa H157 cells. Basal levels of DR5 in WT H157 cells droped from a ratio of 12.7 to 8.9 in DNIKBO H157 cells. More importantly, etoposide induced expression of DR5 with a ratio of 25.2 drops significantly to 6.9 in DNIKBA H157 cells. Up-regulation of DR5 surface expression also correlated with increased surface expression of TRAIL ligand in WT H157 cells treated with etoposide as is seen in. While WT H157 cells treated with etoposide induce their surface expression of TRAIL from 1.0 to 1.6, this induction is lost in DNI $\kappa$ B $\alpha$  H157 cells. Etoposide therefore induces increased surface expression of DR5 and its ligand, TRAIL. This is a NFKB dependent response, as DNI $\kappa$ B $\alpha$  H157 cells lack the ability to increase the surface expression of both DR5 and TRAIL in response to etoposide.

## Gene profiling defines an anti-apoptotic response to genotoxins in H157 cells.

In contradiction to the pro-apoptotic function of NF $\kappa$ B in H157 cells, NF $\kappa$ B activation in several cell types appears to promote cell survival. To define NF $\kappa$ B regulated genes that contribute to pro- or anti-apoptotic signaling in response to genotoxins, gene profiling was conducted with total RNA isolated from WT and DNI $\kappa$ B $\alpha$  H157 cells. Hu6800 Affymetrix gene

chips compared mRNA levels of 7129 genes between WT and DNI $\kappa$ B $\alpha$  H157 cells treated for 0, 12, or 24 hours with 100 $\mu$ M etoposide compared with DMSO treated control cells. The experimental design was to define genes whose expression was either dependent or independent of NF $\kappa$ B transcriptional activity in the presence or absence of etoposide. Gene chip profiling data was analyzed with GeneSpring software (Silicon Genetics). Mathematical normalization and scaling of data was calculated according to the algorithms within both GeneChip and GeneSpring software. Values from the individual time points were averaged and subjected to t-test analysis in order to increase the power of the study with replicate time points. Only those genes whose expression patterns were identical across the combined time points using the mathematical algorithms from both the GeneChip and GeneSpring software were included in the final gene lists.

Fifty-nine NFkB dependent genes were identified by this stringent screening criteria. Interestingly, the number of genes identified as NFkB dependent dramatically increased with etoposide treatment. Several of the etoposide induced genes identified are well defined in their regulation by NFkB. These include cyclin D2, the TRAF2 associated protein A20, manganese superoxide dismutase (SOD2) and the chemokine Groa (MGSA). Other genes of particular interest include members of the relA family and their role in NFkB signaling, such as relB (I-rel) and NFkB2. Two genes having strong anti-apoptotic functions, the inhibitors of apoptosis (cIAP-1 and cIAP-2), are dramatically induced by etoposide treatment. Literature review using PubMed and gene accession numbers reveals that 28% (17/59) of the genes identified as NFkB-dependent have been previously identified as regulated by NFkB. This percentage is likely higher as many genes on the list have no information regarding the role of NFkB in controlling their expression. This experiment is proof of concept that highly specific dominant negative inhibitory proteins such as DNIkB $\alpha$  can be used to accurately define transcriptional changes in gene expression which occur as a result of inhibiting specific signaling pathways.

## NFkB activation regulates the expression of cell survival proteins.

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Several genes' functions argue for an orchestrated survival response of cells exposed to etoposide. Genotoxins such as etoposide, in addition to DNA replication inhibition, also generate oxygen radicals that cause cellular damage and can themselves induce apoptosis. Reactive oxygen species activate NF $\kappa$ B and a compensatory SOD2 expression results in oxygen radical metabolism and protection from further cellular damage. A20 inhibits NF $\kappa$ B signaling and is well characterized in its ability to inhibit TNF-induced apoptosis. A20 expression would be predicted to inhibit NF $\kappa$ B activation and potentially protect cells from genotoxins. The strong induction of cIAP-1 and cIAP-2 would inhibit the caspases that drive cells down the apoptotic pathway.

Individual analysis of gene transcription profiles from GeneChip and GeneSpring mathematical algorithms yielded a larger list with many genes whose protein products are involved in pro- and anti-apoptotic signaling. Genes whose expression was up regulated in an NF $\kappa$ B dependent manner in response to etoposide included the Bcl family members, Bcl-2, Bcl-2-related protein A1, and Bbc3. The expression of several pro-apoptotic genes was also identified as being down regulated in a NF $\kappa$ B-dependent manner following etoposide treatment. Down regulation would correlate with a protective effect on the cell and included the gene for programmed cell death, BCL2-interacting killer, and several members of the TNF receptor superfamily. In contrast to the anti-apoptotic role of NF $\kappa$ B, transcriptional profiles of several genes also support a pro-apoptotic role for NF $\kappa$ B. Genes whose expression profiles are up regulated in an NF $\kappa$ B-dependent manner in response to etoposide included caspase 3 and caspase 4. Similarly the anti-apoptotic proteins trefoil factor 2 and 3 are down regulated by NF $\kappa$ B in response to etoposide. The gene profiles identified in demonstrate that NF $\kappa$ B orchestrates the transcriptional activity of a host of genes whose functions serve opposing regulatory roles in the control of cellular apoptosis. The orchestrated competition of proteins induced by a stress stimulus such as etoposide promotes repair and survival versus commitment to apoptosis. The results clearly define a critical role for NF $\kappa$ B in the competitive determination of cell fate.

### Validation of mRNA expression profiling.

The NFkB dependent changes in mRNA expression were confirmed for four genes by both RNAse protection assays (RPA) and/or relative reverse transcription PCR (RT-PCR). The results demonstrate equivalent mRNA levels for cyclins A, B, C, D1, and D3 in both WT and DNIkB $\alpha$  H157 cells with and without etoposide treatment. WT H157 cells express cyclin D2, which is lost in DNIkB $\alpha$  H157 cells both at rest and with etoposide treatment. Of notable importance, the difference in cyclin D2 mRNA expression between WT and DNIkB $\alpha$  H157 cells without etoposide treatment was not of sufficient magnitude by the initial mathematical screening algorithms to be allowed in the final gene lists, but does appear when screened by the individual algorithms.

RNA levels were also quantitated by Sybr-green RT-PCR analysis of total RNA harvested after 24 hours treatment with etoposide in WT and DNIkB $\alpha$  H157 cells. RNA concentrations were controlled with internal 18S RNA standardized curves. RNA levels of cyclin D2 were down regulated by 11.0 ± 3.5-fold in DNIkB $\alpha$  H157 cells compared with WT cells. Similarly, cIAP-1 and cIAP-2 were down regulated in DNIkB $\alpha$  H157 compared with WT H157 cells by 11.0 ± 3.7 and 37 ± 21-fold, respectively. Relative RT-PCR analysis confirms the NFkB dependent up-regulation of cyclin D2, cIAP-1, and cIAP-2 in response to etoposide treatment. MEK5c regulation was also confirmed by Sybr-green RT-PCR with a 3.5 + 1.2-fold increase in RNA levels in WT H157 compared with DNIkB $\alpha$  H157 cells, confirming MEK5c NFkB-dependent down-regulation in response to etoposide treatment.

### IAP expression promotes H157 cell survival.

Of all the anti-apoptotic proteins induced by etoposide in a NFxB dependent mechanism, the IAPs show the greatest induction. Therefore, we examined whether overexpression of the IAP proteins cIAP-1 and cIAP-2 could inhibit etoposide-induced apoptosis. Expression vectors containing the coding sequences for cIAP-1, cIAP-2, and anti-sense cIAP-1 were generated in plasmids harboring coding sequence for green fluorescent protein (GFP) encoded downstream from an internal ribosomal entry site (IRES).

Genes inserted within this site generate polycistronic messages, which encode both GFP and the gene of interest. H157 cells stably transfected with vector alone, cIAP-1, cIAP-2, and anti-sense cIAP-1 were grown under G-418 selection and screened by flow cytometry for expression of GFP. Stable populations demonstrated GFP expression in 75-80% of the G418 resistant cells. Sybr-green RT-PCR demonstrated overexpression of cIAP-1 and cIAP-2 greater than 10-fold compared with H157 cells harboring empty IRES-GFP vector and wild type H157 cells. The results of apoptosis assays for the various H157 cell lines when exposed to 100  $\mu$ M

etoposide for 24 hours showed wild type H157 cells and H157 cells harboring empty vector had  $23.2\% \pm 1.2\%$  and  $21.4\% \pm 1.4\%$  of the cell population apoptotic following etoposide treatment, respectively. Overexpression of cIAP-1 or cIAP-2 resulted in a significantly inhibited apoptotic response to etoposide with  $10.6\% \pm 2.8\%$  and  $12.2\% \pm 1.8\%$  of the cells undergoing apoptosis, respectively. A similar degree of apoptosis compared with WT H157 was seen in the cIAP-1 antisense vector, IAP-1-AS, demonstrating an apoptotic response of  $23.9\% \pm 1.9\%$  with etoposide treatment. Increased expression of IAPs results in chemo-resistance with a 50% diminution in apoptosis in H157 cells treated with etoposide.

### Conclusions

These findings demonstrate a significant role for surface TRAIL expression in human lung carcinomas, as tumors are deficient in TRAIL mRNA compared with autologous non-tumor lung tissue. Specific alteration of the death receptor pathway proteins (through over-expression of DcR1 or loss of DR5/TRAIL expression) results in attenuated genotoxin response resulting in chemo-resistance. Similarly, over-expression of IAPs results in chemo-resistance. Many groups have defined malignancies, which possess altered surface expression of TRAIL and its receptors. Head and neck cancers often have deleted DR4 alleles, and loss of function mutations often are present in the death domain of the remaining allele. Multidrug resistant leukemia cells alter the death receptor profile and downregulate TRAIL, while gastrointestinal tract tumors have been shown to have increased DcR1. Recent data also suggests that breast cancers have altered DR4 and DR5 expression profiles. Downstream of the death receptors, pediatric neuroblastomas inactivate caspase 8 either by deletion or mutation leading to decreased protein expression. Other malignancies including breast and skin have demonstrated increases in IAP expression, while IAP expression in ovarian carcinoma inversely correlates with cisplatin sensitivity. This work and previous studies indicate that cancers develop aberrant death receptor pathways at all levels of the signaling cascade. Loss of the death receptor entry point into the apoptotic module may endow malignant populations with chemo-resistant capabilities.

TRAIL and DR5 expression are regulated in response to chemotherapy in a NF $\kappa$ B dependent mechanism. Using gene expression profiling reveals an orchestrated pro-apoptotic and anti-apoptotic transcriptional response to chemotherapy. From this analysis it becomes clear why NF $\kappa$ B has been proposed to have an anti-apoptotic function as well as a pro-apoptotic function in different cell types. Our studies stress the essence of a critical balance between NF $\kappa$ B pro- and anti-apoptotic signaling, which might drive a cell towards survival or death. Perturbation of this critical balance in the apoptotic module can reverse the apoptotic signaling set in motion by genotoxin insult. H157 squamous cell carcinoma cells treated with etoposide and doxorubicin chemotherapy up-regulate the transcription and surface expression of DR5 and TRAIL proteins. This up-regulation of death receptor and ligand serves as an entry point in the apoptotic module, which triggers the cell to pursue an apoptotic end despite the expression of counter-acting survival proteins such as cIAP-1 and cIAP-2 survival proteins. While each of these proteins is regulated by NF $\kappa$ B, it is the level of protein expression relative to one another that determines whether NF $\kappa$ B ultimately serves a pro-apoptotic or anti-apoptotic signaling role.

The mechanism by which genotoxin induced expression of NFkB-dependent DR5 and TRAIL overcomes the anti-apoptotic signaling of NFkB-dependent cIAP-1 and cIAP-2 is not entirely clear. A simplistic mechanism by which death receptor signaling predominates over survival signals may relate to the ratio of expression between these opposing proteins. Higher

expression of pro-apoptotic or anti-apoptotic signaling molecules relative to one another may serve to activate or suppress the apoptotic module. More complex mechanisms involving other mediating proteins are readily plausible. It is now apparent that there is cross-talk between the death receptor and mitochondrial apoptotic pathways as exemplified by the identification of the mitochondrial membrane protein, DIABLO, which serves to bind IAP proteins, inactivating their anti-apoptotic role. Etoposide or doxorubicin may additionally activate DIABLO to suppress the IAP signaling and allow the death receptor signal to predominate. While the exact mechanism remains unclear, these studies demonstrate an NF $\kappa$ B-dependent orchestration of signaling proteins with opposing apoptotic functions that ultimately lead to cellular death mediated through death receptors activation of the apoptotic module.

DNA damaging agents activate multiple entry points into the apoptotic module. In addition to the death receptor signal cascade, direct mitochondria injury occurs from oxygen radical generation. Cytochrome c release followed by caspase 9/Apaf-1 activation contributes to the death signal. The integrity of these death pathways counter-balances survival signals concomitantly generated. Tumor cells that develop inactivating mutations in death pathways or increased survival signals with chemotherapy treatment have a survival advantage. We have shown that NFkB regulates the expression of DR5, TRAIL, and IAPs. Inactivation of NFkB enhances tumorigenic potential and chemoresitance, demonstrating that the death receptor component in H157 cells dominates etoposide chemotherapy response. Non-small cell lung cancer is notoriously resistant to chemotherapy with average response rates around 40%. This response rate drops dramatically with second line chemotherapy regimens. Our data suggests that lung carcinomas possess altered TRAIL response rendering them resistant to chemotherapy. Manipulation of the apoptotic module through inhibition of survival proteins or enhancement of death pathways may restore a normal apoptotic response in lung tumors to improve chemotherapy response rates.

### Publications

Spalding, A.C., Jotte, R.M., Scheinman, R.I., Geraci, M.W. and Johnson, G.L. (2002) TRAIL and inhibitors of apoptosis are opposing determinants for NFKB-dependent, genotoxininduced apoptosis of cancer cells. <u>Oncogene</u> 21, 260-271.

www.nature.com/onc

# TRAIL and inhibitors of apoptosis are opposing determinants for NF-*k*B-dependent, genotoxin-induced apoptosis of cancer cells

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Opposing pro- and anti-apoptotic actions of TRAIL and the inhibitors of apoptosis (IAPs) contribute to the cell's decision to survive or die. We demonstrate that in H157 human lung carcinoma cells, etoposide and doxorubicin induce the NFkB-dependent expression of both pro- and anti-apoptotic proteins including TRAIL and its death receptor, DR5, and IAPs. Inhibition of NF-kB activation in H157 cells in response to genotoxin resulted in loss of cell surface expression of TRAIL and DR5, aggressive growth and chemotherapy resistance of tumors in nude mice. Similar to the paracrine TRAIL response in H157 cells, the sensitivity of normal lung and breast epithelium and carcinomas to undergo genotoxin-induced apoptosis correlates strongly with cell surface expression of TRAIL. Suppression of TRAIL signaling by expression of the TRAIL decoy receptor, DcR1, confers chemo-resistance to cancer cells. These findings demonstrate that TRAIL signaling via its death receptors is a significant contributor to genotoxininduced apoptosis in human epithclial carcinomas. Oncogene (2002) 21, 260-271. DOI: 10.1038/sj/onc/

Keywords: TRAIL; IAP; NF- $\kappa B$ ; apoptosis; chemo-

#### Introduction

Apoptosis is characterized as being initiated either by death receptor activation or mitochondrial changes mediated by Bel-2 family members (Yang et al., 1997). Oligomerization of death receptors such as Fas or DR4 and DR5 upon binding FasL or TRAIL leads to caspase 8 activation (Muzio et al., 1996). Activation of pro-apoptotic Bcl-2 family proteins (Bad, Bid, Bax,

Received 8 August 2001; revised 19 September 2001; accepted 9

Bak) results in mitochondrial membrane pore formation, loss of membrane potential and the release of cytochrome c and other mitochondrial proteins (Jurgensmeier et al., 1998; Li et al., 1998; Luo et al., 1998; Zha et al., 1996). Apaf-1 binding of released cytochrome c leads to caspase 9 activation (Zou *et al.*, 1999). Both caspase 8 and caspase 9 can activate caspase 3 whose activation is generally considered the irreversible commitment to apoptosis.

Cytokines such as FasL and TRAIL trigger the apoptosis module using death receptors as their entry point by activating caspase 8 (Chaudhary et al., 1997; Schneider et al., 1997). Toxins such as antimycin A appear to bind to the BH3 domain of Bcl-2 and Bcl- $X_L$ and initiate apoptosis by influencing mitochondrial pore formation and membrane potential (Tzung et al., 2001). In contrast to the selective actions of FasL, TRAIL and antimycin A, other apoptotic stimuli such as genotoxins can activate apoptosis via both death receptors and regulation of Bcl-2 family proteins (Sun et al., 1999). Genotoxins stimulate mitochondrial cytochrome c release via regulation of Bcl-2 and induce the expression of both FasL and TRAIL in different cell types (Ashkenazi and Dixit, 1999; Kasibhatla et al., 1998; Kaufmann and Earnshaw, 2000). The relative contribution of Bcl-2 family proteins and death receptors in genotoxin-induced apoptosis appears to vary depending on the tumor origin and genotoxin.

It is apparent that changes in the expression of death receptors, their ligands and the Bcl-2 family proteins can alter the apoptotic potential of cells. Altered expression or mutation of these proteins provide a survival advantage to tumor cells by down-regulating the entry points that control the activation of apoptosis. We have found with lung and breast-derived epithelial cells, there is a strong correlation between the magnitude of genotoxin-stimulated apoptosis and the expression of TRAIL. Expression of TRAIL and its death receptor, DR5, is regulated in an NF-kB dependent mechanism (Gibson et al., 2000; Ravi et al., 2001). The genotoxins etoposide and doxorubicin strongly activate NF-xB. In concert with controlling TRAIL/DR5 expression, NF-KB regulates the expres-

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sion of several anti-apoptotic proteins. This orchestrated response of pro-apoptotic and anti-apoptotic proteins determines the fate of cells to a genotoxic stress. TRAIL is generally able to overcome the antiapoptotic gene induction in epithelial cells. However, when the TRAIL response system is inhibited, chemoresistance and the NF- $\kappa$ B dependent anti-apoptotic response prevail. Cumulatively, our findings indicate that inhibition of TRAIL signaling is a mechanism for epithelial-derived carcinomas to escape apoptosis in response to genotoxins.

### Results

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# NF- $\kappa B$ activity is necessary for the apoptotic response to etoposide in lung and breast cancer cells

In H157 lung squamous carcinoma the expression of dominant negative IkBa (DNIkBa) inhibits genotoxininduced apoptosis (Figure 1a). To define the importance of NF-kB in etoposide-induced apoptosis, wild type (WT) H157 cells and DNIkBa H157 cells were treated with ctoposide, and apoptosis was quantitated by acridine orange. Basal levels of apoptosis in unstimulated WT H157 cells was approximately  $5\pm1.6\%$  and increased to  $31\pm2.1\%$  and  $42\pm4.7\%$ with 48 h exposure to 30 and 100  $\mu$ M etoposide, respectively (Figure 1a). Apoptosis in unstimulated DNIkBa H157 cells was comparable to WT H157 at  $6 \pm 2\%$ . With 30  $\mu$ M ctoposide, the apoptotic response was suppressed 40% or greater in DNIkBa relative to WT H157 cells (from  $31\pm21\%$  in WT H157 to  $17\pm6.3\%$  for DNI<sub>K</sub>Ba H157 cells seen in Figure 1a, P < 0.005). Similarly, the  $42 \pm 4.7\%$  apoptotic cells observed with 100  $\mu$ M etoposide treatment for 24 h in WT H157 cells decreased to  $20 \pm 4.2\%$  in DNI $\kappa$ Ba H157 cells (P < 0.005). These studies demonstrate that NF- $\kappa$ B activity is required for maximal apoptotic response to etoposide insult in H157 carcinoma cells.

We have observed a similar pro-apoptotic function for NF- $\kappa$ B in other carcinomas from breast and lung. As shown in Figure 1a, ZR-75-1 breast adenocarcinoma cells behave similarly to H157 cells in that DNI $\kappa$ B $\alpha$ inhibits doxorubicin-induced apoptosis. The apoptotic index of WT ZR-75-1 cells rose from  $4\pm1\%$  to  $14\pm3\%$  with 100 nM doxorubicin and  $48\pm6\%$  with 1  $\mu$ M doxorubicin treatment for 48 h. The stable expression of DNI $\kappa$ B $\alpha$  in ZR-75-1 cells did not change basal apoptosis levels of  $4\pm1\%$ . Forty-eight hour treatment of the DNI $\kappa$ B $\alpha$  ZR-75-1 cells with 100 nM doxorubicin did not induce apoptosis ( $4\pm1\%$ , P<0.005 vs WT) while 1  $\mu$ M doxorubicin yielded only 20% of apoptosis found in WT cells ( $13\pm3\%$ , P<0.005 vs WT).

These observations in combination with the role of NF- $\kappa$ B in regulating TRAIL expression (Ravi *et al.*, 2001), led us to examine the role of NF- $\kappa$ B signaling and TRAIL in the control of genotoxin-induced apoptosis in human carcinoma cells. The DNI $\kappa$ B $\alpha$  mutant protein we have used in our studies contains

serine to alanine conversions at residues 32 and 36 that abolish the ability of IKK $\alpha$  to phosphorylate these sites. This prevents ubiquitination and degradation of mutant 1 $\kappa$ B $\alpha$  and subsequent dissociation and nuclear translocation of NF- $\kappa$ B. The inhibition of genotoxin-induced apoptosis was observed in H157 cells stably expressing DNI $\kappa$ B $\alpha$  following transfection and drug selection or acutely after infection with adenovirus encoding DNI $\kappa$ B $\alpha$  (not shown). Because similar results were seen for genotoxin resistance when DNI $\kappa$ B $\alpha$  was expressed by stable transfection or acute adenovirus infection we chose to use stably expressing DNI $\kappa$ B $\alpha$  H157 cells for further study *in vitro* and *in vivo*.

To confirm the dominant negative property of the I $\kappa$ B $\alpha$  mutant, we demonstrated H157 cells expressing DNIkBa fail to stimulate NF-kB DNA binding activity relative to wild type H157 cells following treatment with phorbol cster (PMA) (Figure 1b) or etoposide (Figure 1c). Inhibition of NF-kB DNA binding activity in response to PMA by expression of  $DNI\kappa B\alpha$  is specific, as AP-1 DNA binding activity remains intact in the same nuclear extracts. For etoposide treatment, both WT and DNIkBa H157 cells were treated with genotoxin for 12 and 24 h (Figure 1b), NF-KB DNA binding activity is induced following treatment of H157 cells for 12 and 24 h with etoposide, while  $DNI\kappa B\alpha$ H157 nuclear extracts show no increase in NF- $\kappa B$ DNA binding activity. Etoposide treatment of WT H157 cells also resulted in decreased levels of  $I\kappa B\alpha$ protein detected by immunoblotting, reflective of the phosphorylation, ubiquitination, and protein degradation of  $I\kappa B$  that allows nuclear translocation and DNA binding of NF-KB (Figure 1d). DNIKBA H157 cells retain stable and consistent cytosolic levels of IKB protein despite treatment with etoposide. These assays confirm that  $DNI\kappa B\alpha$  effectively abolishes the stimulation of NF-kB activity, preventing transcriptional activation of NF-kB target genes.

To assess further the biologic implications of NFRB inhibition, WT and DNIkBa H157 cells were examined for tumorigenic potential in nude mouse xenografts. 1×106 WT H157 or DNIkBa H157 cells were injected into nude mice, and tumor volumes were followed for 31 days at which time tumor mass necessitated animal sacrifice. WT and DNIkBa H157 mouse xenografts grew comparably until day 17, when separation of tumor growth curves became apparent (Figure 1e). DN1 $\kappa$ B $\alpha$  H157 xenografts grew to a larger size  $(450\pm60 \text{ mm}^3, n=38)$  over a shorter time frame compared to WT H157 xenografts  $(223 \pm 20 \text{ mm}^3)$ n=38) with growth differences reaching statistical significance (P < 0.005) at day 17 that persisted until the end of the experiment. At day 31, the DNI $\kappa B\alpha$ H157 xenografts reached a size of  $2426 \pm 658 \text{ mm}^3$ while WT H157 xenografts had grown to  $1233 \pm 218 \text{ mm}^3$  (P < 0.005).

The aggressive tumor growth characteristic of  $DNI\kappa B\alpha$ H157 xenografts is not simply the result of a faster growth rate compared with WT H157 cells. In fact, *in* vitro growth curves suggest that  $DNI\kappa B\alpha$  H157 cells

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Figure 1 Loss of NF-xB results in chemo-resistance and aggressive tumor growth. (a) Loss of NF-xB activity results in a blunted apoptotic response to etoposide and doxorubicin in a dosc-dependent manner. We treated WT H157 cells or DNIkBa H157 cells with ctoposide for 48 h and then measured apoptosis. WT or DNIkBa ZR-75-1 cells were treated with the indicated doses of doxorubicin for 48 h. Data points represent the means of duplicate samples from three independent experiments while error bars are s.c.m. (b) DNI&Ba effectively abolishes NF-&B nuclear translocation. Gel shift assays were done on nuclear lysates from WT and DNIkBa H157 in the absence of presence of PMA stimulation. WT H157 cells demonstrate inducible NF-kB DNA binding activity in unstimulated and stimulated cells; no NF-xB DNA binding activity can be demonstrated in DNIxBa nuclear extracts. Binding is specific, as binding is unaffected by AP-1 cold competitor, while NF-xB cold competitor abolishes protein binding to labeled probe (lancs 3,4). Loss of NF-KB DNA binding activity is not the result of a global loss of nuclear protein DNA binding activity, as AP-1 DNA binding activity is conserved in DNIkBa nuclear extracts (lanes 7,8). (c) DNIkBa expression attenuates etoposide induced NFκB translocation to the nucleus. Etoposide induces NF-κB nuclear translocation, which persists to 24 h (lancs 4,5). No NF-κB DNA binding activity can be demonstrated in DNI $\kappa$ B $\alpha$  with etoposide treatment (lanes 7,8). (d) Lack of NF- $\kappa$ B DNA binding activity in DNI $\kappa$ B $\alpha$  H157 cells correlates with stable levels of I $\kappa$ B $\alpha$  protein in cytosolic extracts. Western blotting with an I $\kappa$ B $\alpha$  antibody that recognizes both WT and dominant negative IxBa demonstrates that lxBa levels drop with etoposide treatment as a result of protein dissociation from NF-xB and subsequent ubiquitination and degradation. This fails to occur in DNIxBa H157 cells. (e) Loss of NF-KB activity in DNIKBA H157 cells results in more rapid and more aggressive tumor growth in nude mouse xenografts. We injected DNIxBa and WT H157 cells into the posterior flanks of nude mice to assess in vivo growth characteristics. DNIxBa H157 cells grow more rapidly and to a larger maximal volume over a growth period of 31 days with P decreasing below 0.005 by day 17. Means are from volumes of 38 individual tumors while error bars are s.e.m. (f) The aggressive growth pattern of DNIkBa H157 cells in nude mouse xenografis is not the result of a shortened cell cycle. Growth curves for WT H157, DNIkBa H157 and vector alone H157 cells are comparable, demonstrating that the accelerated rate of growth and aggressive tumor phenotype of DNIkBa H157 cells in nude mouse xenografis is not the result of an altered cell cycle. Means displayed are from duplicate samples of two independent

grow with a comparable but somewhat slower doubling time relative to WT H157 cells (Figure 1f).

# NF-KB is required for genotoxin but not paclitaxel-induced apoptosis

Treatment of WT H157 cells with 300 nM doxorubicin resulted in 32% apoptosis over 48 h. This is inhibited by almost 50% in DNI $\kappa$ B $\alpha$  H157 cells with an apoptotic index of 17% after a similar treatment with doxorubicin (not shown). This is comparable apoptotic suppression as was seen with 100  $\mu$ M etoposide treatment (Figure 1a). However, to determine the specificity of NF- $\kappa$ B in genotoxin induced apoptosis, we treated cells with the microtubule toxin paclitaxel. Paclitaxel induces apoptosis primarily through phosphorylation and subsequent inactivation of Bcl-2. The phosphorylation renders Bcl-2 unable to sequester proapoptotic Bcl-2 family members such as Bax which induce mitochondria release of cytochrome c (Srivastava et al., 1999; Strobel et al., 1996). Figure 2a shows the response of WT and DNI $\kappa$ B $\alpha$  H157 cells exposed to 30 nM and 300 nM paclitaxel for 24 h. Fifty to 52% of WT H157 cells are apoptotic after 24 h for both paclitaxel doses while 40% and 46% of DNI $\kappa$ B $\alpha$  H157 cells are apoptotic after 30 and 300 nM paclitaxel treatment, respectively. Paclitaxel-induced apoptosis was not significantly dependent on NF- $\kappa$ B activation, whereas loss of NF- $\kappa$ B activation in DNI $\kappa$ B $\alpha$  expressing cells resulted in significant resistance to both etoposide and doxorubicin.

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In agreement with the in vitro results, analysis of xenografis in nude mice indicated sensitivity of WT H157 and DNIkBa H157 cell-derived tumors to treatment with paclitaxel (Figure 2b,c). The result with paclitaxel is in sharp contrast to the etoposide resistance of DNI $\kappa$ Ba H157 cells in vitro as well as in xenografts. Wild type H157 xenograft growth is suppressed with etoposide treatment with a separation of growth curves reaching statistical significance by day 21 (Figure 2d). In contrast, DNIkBa xenografts are resistant to etoposide (Figure 2e). Despite treatment with etoposide, DNIkBa xenografts continue to grow at the same rate as untreated DNIkBa expressing tumors. These findings indicate that inhibition of NF- $\kappa B$  function in H157 cells results in a more aggressive tumor that is refractory to treatment with etoposide,

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similar to the anti-apoptosis and chemo-resistance of  $DNI\kappa B\alpha$  expressing H157 cells assayed in vitro (Figure 1a).

# Etoposide induces surface expression of TRAIL and DR5

Increased cell surface expression of TRAIL is a general response to etoposide in both lung and breast epithelium (Figure 3). Using non-transformed, nonimmortalized primary human airway epithelium (HAEC) and primary human mammary epithelium (HMEC) in conjunction with A549 lung adenocarcinoma, MDA-231 breast carcinoma, and ZR-75-1 breast caricinoma cells, we measured cell surface expression of TRAIL in response to 24 h of 100  $\mu M$ etoposide. All five unstimulated cell lines had no detectable surface TRAIL expression (left column). Reproducible increases of 1.6-3.1-fold (middle and right column) were demonstrated after etoposide treatment. Bar graphs display the relative fluorescence for each cell line with and without etoposide (right column).

The chemo-resistance of DNI $\kappa$ B expressing H157 cells in vitro and in vivo indicates a major change in apoptotic potential resulting from loss of NF- $\kappa$ B signaling. We had previously shown that TRAIL and DR5 expression in different cell types was NF- $\kappa$ B



Figure 2 NF- $\kappa$ B inhibition causes genotoxin-specific chemo-resistance in lung tumors. (a) DNI $\kappa$ B $\alpha$  does not protect against the microtubule toxin paclitaxel. Treatment of WT and DNI $\kappa$ B $\alpha$  H157 cells for 24 h with the indicated doses of paclitaxel induces apoptosis to similar levels. Means displayed are from duplicate samples of three independent experiments. To validate these results, we xenografted WT and DNI $\kappa$ B $\alpha$  H157 cells into nude mice and treated with etoposide or paclitaxel. Arrows indicate days of treatment with paclitaxel (b and c) or etoposide (d and c). (b) WT H157 xenografts fail to grow when treated with paclitaxel blunted tumor growth curves (P < 0.005 by day 21, n = 20 tumors). (c) DNI $\kappa$ B $\alpha$  H157 xenografts also respond to paclitaxel chemotherapy with growth curves (P < 0.005 by day 21, n = 20 tumors). (d) Etoposide treatment of WT H157 xenografts inhibits tumor and etoposide treated animals, n = 20 tumors). DNI $\kappa$ B $\alpha$  xenografts demonstrate etoposide (P = NS between vehicle and etoposide treated animals, n = 20 tumors). DNI $\kappa$ B $\alpha$  xenografts demonstrate etoposide chemo-resistance

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Figure 3 Etoposide induces surface expression of TRAIL. Primary human manumary (HMEC) and lung epithelial cells (HAEC). A549 and H157 lung carcinoma cells, MDA-231 and ZR-75-1 breast carcinoma cells were analysed for TRAIL surface expression in response to 24 h of 100  $\mu$ M etoposide treatment. The left column indicates cell surface expression of TRAIL (blue line) versus background fluorescence (black line) of unstimulated cells. The middle column contains histograms of TRAIL surface expression (red line) compared to background fluorescence (black line) after 24 h of etoposide treatment. Relative fluorescences for each cell type in unstimulated (blue bars) and etoposide treated (red bars) are depicted in the graphs in the right column. All signals are normalized to background and set to one; therefore a relative fluorescence of one indicates no detectable surface protein

dependent (Gibson *et al.*, 2000), therefore, we predicted that a loss in the surface expression of TRAIL or its death promoting receptors, DR4 and DR5, could account for the loss in genotoxin sensitivity we observed in DNI $\kappa$ B expressing H157 cells. To test this hypothesis, WT and DNI $\kappa$ B $\alpha$  H157 cells were treated with 100  $\mu$ M etoposide for 24 h and subjected to flow

cytometric analysis for measurement of DR4, DR5 and TRAIL cell surface expression. Figure 4a and b depict the surface expression of DR4 in WT H157 and DN1 $\kappa$ Ba H157 cells with and without etoposide treatment. Figure 4c shows this data in bar graph format in order to display the relative DR4 expression with and without etoposide treatment of cells. WT H157 cells

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demonstrate DR4 surface expression of  $15.3 \pm 0.6$  and  $18.1 \pm 1.7$  (n=3, P < 0.05) times greater than background with and without etoposide treatment, respectively. No significant difference in DR4 surface expression is seen in DNI $\kappa$ B $\alpha$  H157 cells, which demonstrate comparable DR4 surface expression ratios of  $12.2 \pm 0.9$  and  $17.0 \pm 0.5$  (n=3, P < 0.05) with and without etoposide treatment, respectively. The levels of DR4 expression correlates with RNase protection assays measuring DR4 transcript (data not shown). Although DR4 surface expression levels decrease in both WT and DNI $\kappa$ B $\alpha$  H157 cells in response to etoposide, the magnitude of change being less than 50% stands in direct contrast to DR5.

DR5 surface expression increases with etoposide treatment in WT H157 cells, and this induction of surface expression is inhibited in DN1 $\kappa$ B $\alpha$  H157 cells (Figure 4d, e, and f). Basal levels of DR5 in WT H157 cells drop from a relative surface expression of 13.1±0.5 in wild type to 9.2±0.3 (n=3, P<0.001) in DN1 $\kappa$ B $\alpha$  H157 cells. More importantly, etoposide induced expression of DR5 with a surface expression ratio of 24.5±2.3 in wild type cells drops significantly AC Spaiding et al

to  $6.8 \pm 0.2$  (n=3, P<0.0005) in DN1 $\kappa$ Ba H157 cclls (Figure 4f).

TRAIL surface expression in WT H157 cells treated with etoposide is also up-regulated as seen in Figure 4g and i. While WT H157 cells treated with etoposide induce surface expression of TRAIL 1.6-fold, this induction is absent in DNI $\kappa$ B $\alpha$  H157 cells (Figure 4h and i). The surface expression of TRAIL is relatively low but highly reproducible in its induction by etoposide (n=3, P<0.01). Etoposide therefore induces increased surface expression of TRAIL and one of its death promoting receptors, DR5. This is a NF- $\kappa$ B dependent response, as DNI $\kappa$ B $\alpha$  H157 cells lack the ability to increase the surface expression of both TRAIL and DR5 in response to etoposide.

### Profiling the genotoxin response of H157 cells

NF- $\kappa$ B activation in several cell types appcars to promote cell survival not apoptosis as observed with H157 cells (Baeuerle and Baltimore, 1996; Basu *et al.*, 1998; Lin *et al.*, 1998). The expression of the inhibitors of apoptosis, or IAPs, inhibit specific



Figure 4 NF- $\kappa$ B induces the surface expression of DR5 and TRAIL. To determine if NF- $\kappa$ B mediates changes in DR4, DR5, or TRAIL surface protein expression, flow cytometry was conducted on WT (a, d, g) or DNI $\kappa$ Ba H157 cells (b, e, h) to assess for DR4 (a, b), DR5 (d, e), and TRAIL (g, h) proteins. Solid black lines represent control background fluorescence; dashed black lines represent eloposide background fluorescence. Untreated cells (blue lines) were compared with cells treated with ecoposide for 24 h at relative fluorescence indicative of surface protein. This flow cytometry data demonstrates that etoposide induces the surface of DR5 and TRAIL in WT H157 cells (f, i). DNI $\kappa$ Ba cells demonstrate a diminished basal level of DR5 as well as a loss of DR5 induction with etoposide treatment (f). Etoposide-induced TRAIL expression is also lost in DNI $\kappa$ Ba cells compared with error bars demonstrating s.c.m.

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caspases and have been shown to be positively regulated by NF-kB. Thus, in addition to TRAIL and DR5, NF-xB is capable of regulating pro-survival genes. To define NF-xB regulated genes that contribute to pro- or anti-apoptotic signaling in response to genotoxins in cancer, gene profiling was conducted with total RNA isolated from WT and DNI<sub>K</sub>Ba H157 cells. H157 cells stably expressing  $DNI\kappa B\alpha$  and DNIkBa-adenovirus infection of H157 cells were used for analysis with similar results in profiling the gene expression response to etoposide treatment. Hu6800 Affymetrix gene chips compared mRNA levels of 7129 genes between WT and DNIxBa expressing H157 cells treated for 0, 12, or 24 h with either 100  $\mu$ M etoposide or DMSO treated control cells. Using this approach we were able to define genes whose expression is dependent on NF-xB transcriptional activity in the presence or absence of ctoposide.

Figure 5 presents the list of genes regulated in a NF**kB** dependent mechanism in response to etoposide. Database review revealed that 33% (17/52) of the 52 genes identified as NF-kB-dependent have been previously identified as regulated by NF-KB. This percentage is likely higher as many of the genes have no information regarding the role of NF-kB in controlling their expression. From a total of 52 genes dependent on NF-kB activation for regulation of their expression, 13 etoposide inducible genes were readily identified that have previously characterized functions that can clearly promote or inhibit apoptosis (Figure 5a). The values listed in Figures 5a and b indicate the etoposide induced fold change in WT cells that was absent in DNIkBa expressing cells. Two etoposide induced genes, A20 (Cooper et al., 1996; Krikos et al., 1992) and manganese superoxide dismutase (SOD2) (Jones et al., 1997) are well defined in their regulation by NF-kB. Three genes having strong anti-apoptotic functions, the inhibitors of apoptosis (cIAP-1, cIAP-2 and XIAP), are induced by etoposide treatment. Forkhead FREAC-1 is a lung specific forkhead transcription factor and whose mRNA was increased sixfold in response to etoposide in an NF-xBdependent mechanism (Hellqvist et al., 1996; Mahlapuu et al., 1998; Pierrou et al., 1994). Specific forkhead transcription factors have been implicated in regulating pro-apoptotic gene expression including Fas ligand (Brunet et al., 1999). Trefoil factor is a cytokine known to promote cell survival (Chen et al., 2000; Suemori et al., 1991; Taupin et al., 2000; Thim, 1989), as is cell adherence to the extracellular matrix protein, fibronectin (Sakai et al., 2001; Zhang et al., 1995). The expression of both trefoil factor and fibronectin was NF-kB dependent and markedly inhibited in response to etoposide. Overall, of the 13 genes whose function has been clearly associated with survival and/or apoptosis, 11 were up-regulated and two downregulated in response to etoposide in a NF-KB dependent mechanism,

Granted many other genes are regulated in response to etoposide (Figure 5b) or by NF- $\kappa$ B independent of etoposide (Figure 5c) that may

sented in the array may have significant roles in the response to genotoxin. Despite these limitations, the gene profiles identified in Figure 5a argue for an orchestrated response of cells exposed to etoposide that promotes survival versus the paracrine death response mediated by TRAIL. Genotoxins such as etoposide, in addition to inhibiting DNA replication, also generate oxygen radicals that cause cellular damage and can themselves induce apoptosis (Verhaegen et al., 1995). Reactive oxygen species activate NF-kB (Wang et al., 1999) and a compensatory SOD2 expression results in oxygen radical metabolism and protection from further cellular damage. A20 inhibits receptor activation of NF-xB and is well characterized in its ability to inhibit TNF-induced apoptosis (Opipari et al., 1992). A20 expression would be predicted to protect cells from genotoxins by suppressing death receptor signaling. The strong induction of cIAP-1 and cIAP-2 would inhibit the caspases that drive cells down the apoptotic pathway (Chu et al., 1997; Wang et al., 1998). In contrast, the induction of TRAIL and DR5 would stimulate caspases that drive the apoptotic response. Induction of TNF receptor expression may also contribute to a pro-apoptotic response. Clearly, the NF-xB-dependent response to etoposide is complex and shows a response that allows cells to survive or die. NF-kBdependent gene expression in response to etoposide is neither pro-survival nor anti-survival but rather allows a decision process for cell fate.

regulate cell survival. In addition, genes not repre-

### Validation of mRNA expression profiling

The NF-KB dependent changes in mRNA expression were confirmed for four gencs, two with clear functions in apoptosis and two in controlling cell cycle or signaling. cIAP-1 and 2, cyclin D2 and MEK5c expression was monitored by relative reverse transcription PCR (RT-PCR). Results shown in Figure 6 depicts the fold-changes in RNA levels by Sybr-green RT-PCR analysis of total RNA harvested after 24 h treatment with ctoposide in WT and DNIkBa H157 cells. RNA concentrations were controlled with internal 18S RNA standardized curves. The induction of cIAP-1 and cIAP-2 was blocked in DNIkBa H157 cells compared with WT H157 such that WT cells had  $11 \pm 3.7$  and  $37 \pm 21$ -fold higher levels of cIAP-1 and cIAP-2 respectively. Changes in expression of cyclin D2 and MEK5c were similarly confirmed, indicating the general validity of the expression profiles shown in Figure 5.

### IAP expression promotes H157 cell survival

Because IAPs are strongly induced by genotoxin treatment, we chose to examine whether expression of the IAP proteins cIAP-1 and cIAP-2 could inhibit etoposide-induced apoptosis. Expression vectors containing the sequences for cIAP-1 and cIAP-2 were generated in IRES vectors encoding green fluorescent Α

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# Etoposide regulated genes associated

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Etoposide Induced Fold			
Change in WT H157 Cells	Gene Name		
27	CIAP-I		
£	CIAP-2		
# 3	Forkhead transcription factor FREAC		
9.3	TNFR		
3.4	NFkB2		
3	DB5*		
2.7	420		
2.5	Balls		
24	Fiel B		
	Manganese superoxide dismutase		
2	XIAP		
2	TRAIL		
42	Fibropectie		
-30	Trafoil factor		

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### Other etoposide regulated genes

EIODOSIOE Induced Fold	genes			
Change In WT H157 Cella	Gene Name	Etoposide Induced Fold		
	ICAM-1	Chiange in W1 115/ Cells	Gena Name	
14.3	Cyclin D2	-23.7	RGS4	
12.8	MDA-7	-14.3	MEK5c	
9	Pentario	-6.7	C1 inhibitor	
9	Groc	-5.1	Gelsolin	
6.4	Osteonidogen	-3.7	Apolipoprotein E	
6.2	B94 protein		IFNy receptor 2	
4,6	HAEV107 tumor suppressor	-3.6	Carbonic anhydrase IX	
4.5	Bloom syndrome protein	-3.5	Dual specificity phosphatase 4	
4.3	GTP cyclohydrolaae	-3.1	IRS-1	
2.8	CD3-associated protein		Plectin	
2.5	E6-AP oncogene	-2.8	Transgelin	
2.4	Homeotic protein Pl 2	-2.8	Histone H4	
2	BRCA1 associated protein	-2.6	NADH ubiguinone oxidoreductase	
-		-2.3	Sonthin 1	
		-2.3	Giloma pathogenesis related protein	
		*2.2	CRABP //	

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# NFkB-regulated genes in the absence of etoposide

WT/DNikB H157 cells	Gene Name	Fold change; ratio	
3	Cytidine deaminase	-10.5	Gene Name
2.6	IL-11 MHC class 1 polypeptide	-6.8	Collagen type XV Predicted osteoblast protein
2.6	TEA domain family member 4	-6.6	GS3955 serine/threonine kinase
2	KIAA0018 gene product		

Figure 5 Gene-profiling identifies NF- $\kappa$ B as a regulator of both pro- and anti-apoptotic signaling. Only genes whose expression was similar across all three time points and whose fold change was two or greater by both GeneChip and GeneSpring mathematical algorithms are included in the final gene lists. (a) Gene profiling identifies important apoptotic mediators whose expression is chip. RNase protection assays with probes for TRAIL and DR5 were performed on the RNA samples and quantitated in response to etoposide. (b) Etoposide also altered expression of other genes in an NF- $\kappa$ B dependent manner. (c) NF- $\kappa$ B also regulates genes independent proteins with both pro- and anti-apoptotic roles in response to genotoxin insult

protein (GFP) for selection by flow cytometry. H157 cell populations selected for GFP expression demonstrated overcxpression of cIAP-1 and cIAP-2 greater than 10-fold compared with H157 cells harboring empty IRES-GFP vector and wild type H157 cells as determined by Sybr-green RT-PCR (data not shown). Figure 7 shows the results of apoptosis assays for the various H157 cell lines when exposed to 100  $\mu$ M etoposide for 24 h. Wild type H157 cells and H157 cells harboring empty vector had 23±1.2% and 21±1.4% of the cell population apoptotic following etoposide treatment, respectively. Overexpression of

cIAP-1 or cIAP-2 resulted in a significantly (P < 0.05) inhibited apoptotic response to etoposide with  $10 \pm 2.8\%$  and  $12 \pm 1.8\%$  of the cells undergoing apoptosis, respectively. Thus, increased expression of IAPs results in a 50% diminution in apoptosis in H157 cells treated with etoposide. The results indicate that induction of IAPs would have a survival function in response to etoposide. Etoposide therefore induces expression of several genes that influence cell survival iucluding TRAIL and its death receptor, DR5, and IAPs. The cumulative action of these proteins dictate cell survival.

Олсоgene



Figure 6 Gene profiling validations confirm the importance of endogenous apoptotic mediators in genotoxin-induced apoptosis. To further validate identified gene profiles, Sybr-green relative RT-PCR was performed with total RNA obtained from WT H157 and DN1×Ba H157 cells using primers corresponding to cyclin D2, clAP-1, clAP-2 and MEKSc. The graph represents the mean RNA fold change between WT H157 and DN1×Ba H157 cells treated with etoposide from two independent experiments performed in triplicate while error bars are s.e.m.



Figure 7 NF- $\kappa$ B-mediated induction of DR5/TRAIL overcomes the endogenous activity of cIAP-1 and cIAP-2. H157 cells stably expressing cIAP-1 or -2 were made to determine if IAP over expression counteracts the death receptor apoptotic response to etoposide. WT H157 cells or H157 cells stably harboring IRES vector alone, cIAP-1, or cIAP-2 expression vectors were treated with or without etoposide and apoptosis was quantitated. Expression of cIAP-1 and -2 blunted the apoptotic response of H157 cells to etoposide by 50%. Data bars indicate the mean of three independent experiments performed in duplicate while error bars are s.e.m.

# Suppression of TRAIL death receptor signaling leads to chemo-resistance

Induction of TRAIL expression in response to genotoxins strongly correlates with the magnitude of apoptosis that is observed in normal lung and breast epithelium and carcinoma (Figure 8a). Cell surface TRAIL expression correlates with the apoptotic response to ctoposide for primary human airway epithelium, H157 lung squamous carcinoma cells, A549 lung adenocarcinoma cells, ZR-75-1, and MDA-231 human breast adenocarcinoma cells. To test the prediction that cell surface TRAIL expression is an important autocrine or paracrine response to genotoxin-induced cell death, we

stably expressed in H157 cells the GPI-linked decoy receptor, DcR1, which binds TRAIL but does not signal caspase activation (Sheridan et al., 1997). DcR1 expression functions to bind TRAIL and effectively block TRAIL-induced apoptosis. The etoposide dose response curves seen in Figure 8b demonstrate that as the dose of genotoxin increases, the degree of apoptosis also increases in WT H157 cells. With etoposide doses ranging from  $1-100 \ \mu M$  with 48 h treatment, WT H157 cells displayed apoptotic responses ranging from 18-52%. Two separate stable clones of H157 cells expressing DcR1, as confirmed by flow cytometry analysis of cell surface expression (data not shown), suppress etoposide-induced apoptosis relative to control H157 cells. H157 cells expressing DcR1 suppress etoposide induced apoptosis rates by greater than 50% to levels of 6-24% over the same dose range used with WT H157 cells (n=3, P<0.05 at all doses). DeR1 expression has a strong protective effect against etoposide induced cell death, consistent with a significant role for TRAIL in the death response and diminished TRAIL signaling in chemo-resistance to ctoposide. Figure 8c demonstrates that DcR1 protects against doxorubicin induced apoptosis as well. WT H157 cells increased from  $4\pm1\%$  to  $35\pm3\%$  apoptosis with 300 nM and  $37\pm2\%$  apoptosis with I µM doxorubicin treatment. Two separate stable clones of H157 cells expressing DcR1 suppressed the doxorubicin induced apoptosis at 300 nM ( $22\pm0\%$  for clone 1, 11  $\pm$  4% for clone 2, P < 0.05 vs WT) and at 1  $\mu$ M  $(20 \pm 1\%$  for clone 1,  $16 \pm 4\%$  for clone 2, P < 0.05 vs WT). A similar effect of DcR1 is seen in MDA-231 breast adenocarcinomas (not shown), demonstrating the protective role of DcRI against genotoxins is a general function of the decoy receptor and not a unique response in H157 cclls. The protective effect of DcR1 expression is selective for genotoxins such as etoposide and doxorubicin and is not seen with paclitaxcl-induced H157 cell death (Figure 8d), confirming a significant role for TRAIL expression in the apoptotic response to specific genotoxins but not microtubule toxins. Paclitaxel induced apoptosis to similar levels in WT and two separate DcR1 clones; 30 nM induced  $52\pm6\%$  in WT vs 58  $\pm$  1% in clone 1 vs 47  $\pm$  1% in clone 2 while 300 nM induced  $52\pm4\%$  in WT vs  $62\pm1\%$  in clone 1 vs  $51\pm5\%$ in clone 2.

#### Discussion

Two different but converging experimental lines of study are defining the importance of TRAIL-mediated death signaling in cancer. Several studies have begun to define mutations in the death receptors for TRAIL in different cancers. In metastatic breast cancer (Han *et al.*, 2001), head and neck squamous cell cancer (Fisher *et al.*, 2001; Ozoren *et al.*, 2000; Pai *et al.*, 1998), lung cancer (Fisher *et al.*, 2001; Lee *et al.*, 1999; Wu *et al.*, 2000), and non-Hodgkin's lymphoma (Lee *et al.*, 2001) mutations have been defined in the death receptors for TRAIL. In numerous other cancers changes in the expression profile of TRAIL death receptors has also



Figure 8 TRAIL mediates genotoxin-specific apoptosis. (a) Genotoxin-induced apoptosis correlates with TRAIL surface expression. Primary human lung airway epithelial cells (HAEC), A549 and H157 lung carcinoma cells, MDA-231 and ZR-75-1 doxorubicin. A linear correlation between surface expression and degree of apoptosis with and without coposide or  $R^2 = 0.903$ , demonstrating that genotoxins induce TRAIL expression of TRAIL and per cent apoptosis is seen in these cell lines with expression of DcR1 in H157 cells results in chemo-resistance to etoposide-induced apoptosis in a dosc-dependent fashion. WT H157 represent the means of duplicate samples from two independent experiments while error bars are s.e.m. (c, d). DcR1 expression doxorubicin-induced apoptosis, but is unable to inhibit paclitaxel (d). Apoptosis assays demonstrate that DcR1 suppresses induced themo-resistance is specific for etoposide and doxorubicin. The apoptotic pathway affected by DcR1-for e and d, means are from duplicate samples of experiments done on three separate occasions while error bars are s.e.m.

been defined. Cumulatively, these studies indicate that expression or function of receptors that signal TRAILinduced apoptosis is altered at frequencies approaching 50% in some cancers. Although the significance of these mutations is presently unclear, their frequency and potential survival advantage suggests they may contribute to tumorigenesis and possibly metastasis. TRAIL also has been shown to be decreased in oligodendrogliomas (Nakamura et al., 2000). Our work and that of others are defining an involvement of a paracrine action for TRAIL-induced apoptosis in chemotherapy (Altucci et al., 2001; Gibson et al., 2000). Paracrine TRAIL-induced apoptosis is important for genotoxins like etoposide and doxorubicin but less important for the apoptotic response to microtubule toxins. Mutations that inhibit TRAIL receptor signaling or diminish TRAIL expression should promote resistance to chemotherapy.

What is the evidence that a paracrine TRAIL-induced apoptosis response is important in chemotherapy? Flow cytometric analysis clearly demonstrates that etoposide

and doxorubicin stimulate cell surface expression of TRAIL in normal epithelium and multiple cpitheliaderived carcinoma cell lines. The use of a soluble DR4:Fc fusion protein that binds TRAIL effectively suppresses genotoxin-induced apoptosis (Gibson et al., 2000). This finding demonstrates that surface TRAIL expression contributes to genotoxin-induced cell death. Similarly, reovirus-induced apoptosis involves expression of TRAIL in epithelial-derived tumor cell lines that is inhibited by DR4:Fc fusion proteins or anti-TRAIL antibodies (Clarke et al., 2000). In both studies the use of TNFa or Fas ligand protein traps or antibodies did not inhibit cell death, demonstrating the specificity of TRAIL in both genotoxin and virus-induced apoptosis. We have also used an alternative strategy, namely the expression of the TRAIL decoy receptor, DcR1, to inhibit genotoxin but not paclitaxel-induced apoptosis. DcR1, DR4:Fc and anti-TRAIL antibody suppression of genotoxin-induced apoptosis all demonstrate the importance of cell surface expression of TRAIL in specific apoptotic responses in epithelial-derived carcinomas. Interestingly, both genoAC Spalding of al

toxin- and recovirus-induced expression of TRAIL is dependent on functional NF- $\kappa$ B signaling and inhibited by DNI $\kappa$ B. It was also recently shown that retinoic acidinduced apoptosis of acute promyelocytic leukemia cells is mediated by a paracrine TRAIL action (Altucci *et al.*, 2001). Cumulatively, the findings indicate paracrine TRAIL-induced apoptosis is a major contributing mechanism to the action of different drugs and viralinduced apoptosis of tumor cells.

Profiling the NF-xB-dependent response to etoposide demonstrates the induction of many genes including TRAIL and the IAPs. The IAPs are markedly induced in response to genotoxins and have the ability to significantly inhibit etoposide-mediated cell killing. We did not observe a significant change in expression of Bcl-X<sub>L</sub> or any other Bcl-2 family member in H157 cells. Furthermore, the use of paclitaxel, a microtubule toxin that induces apoptosis primarily through phosphorylation and inactivation of Bcl-2 (Srivastava et al., 1999; Strobel et al., 1996), did not depend on NF-KB activity for initiating programmed cell death. At least in H157 lung squamous carcinoma cells, our studies would argue that NF-xB-dependent IAP expression is a significant mechanism for the survival function of NF-xB signaling. These findings indicate that activation of NF- $\kappa B$  in response to genotoxins stimulates a TRAIL death response and an IAP survival response. In different cancers, the magnitude of TRAIL expression for paracrine-mediated apoptosis versus IAP expression for survival is going to influence the chemo-sensitivity of the tumor. For example, tumor cells that lose the TRAIL paracrine response may be particularly chemo-resistant. Clinically, the administration of recombinant TRAIL concurrent with genotoxin based chemotherapy may help tilt the balance of NF-×B mediated signaling towards tumor cell apoptosis.

#### Materials and methods

### Generation of stable clones

The cDNA for DNIkBa was excised from a CMV hikBa 32S/32A vector and cloned into the HindIll/XbaI sites of pcDNA3.1(+). H157 cells were transfected with lipofectamine (Gibco BRL) and selected for neomycin resistance. Clones were screened for IxBa over-expression by Western blot and gel shift assay for loss of NF-KB DNA binding with nuclear extracts from cells treated with and without PMA. DcRJ stable cell lines were similarly generated with pcDNA3.1(+)/DcR1. After the clones grew to acceptable numbers, flow cytometry for surface expression of DcR1 was performed to verify G418 resistant clones over-expressing DcR1, cIAP-1, cIAP-2, and cIAP-1-AS stable clones were generated by cloning into the EcoR1 site of IRES vector (Clontech), selecting for G418 resistance, and performing flow cytometry to confirm GFP expression. IAP expression was confirmed by RT-PCR.

#### Acridine orange stuining

H157 and DNI $\kappa$ Ba H157 cells were treated with 100  $\mu$ M etoposide, 30 nM paclitaxel, 300 nM paclitaxel, or 1 mM

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doxorubicin. At the given time points, 50  $\mu$ l of a PBS solution containing 100  $\mu$ g/ml acridine orange and 100  $\mu$ g/ml ethidium bromide was added to pelleted cells. Cells were visualized on a fluorescent inverted microscope using a 40 × LWD lens and a high pass FITC filter to allow for nuclear visualization. At least 300 cells per plate were counted in a double blinded fashion. Only cells with both condensed chromatin and membrane blebbing were scored as apoptotic.

### Flow cytometry for detection of surface expressed TRAIL, DR4. DR5 and DcR1

Anti-TRAIL, anti-DR4, anti-DR5, and anti-DcR1 antibodies were a kind gift from Immunex corporation. Flow cytometry was performed as previously described (Sedger *et al.*, 1999). Cells were analysed using a FACScan flow cytometer (Becton Dickinson) running Cell Quest Software (Becton Dickinson).

#### Western blotting

Western blotting was performed as previously described (Gibson *et al.*, 2000). I $\kappa$ B $\alpha$  antibody was used at a concentration of 1:500 (Santa Cruz).

### Xenograft implantation

 $1 \times 10^6$  cells per 100  $\mu$ l 1×PBS and 100  $\mu$ l growth factor reduced matrigel were injected on athymic nude mice postcrior flanks. Tumor volumes were measured twice a week, and tumor volumes were calculated using the formula volume = length<sup>2</sup>\*width\* $\pi/6$ . Treated mice were given 15 mg/ kg etoposide or 15 mg/kg paclitaxel intraperitoneally on days 7, 14 and 21.

### Affymetrix gene chip profiling

Total RNA was isolated per manufacturer's recommendation with RNAzol (Tel-Test) from WT H157 cells and DNI $\kappa$ Ba H157 cells treated for 0, 12, or 24 h with 100  $\mu$ m etoposide (Sigma chemicals). Probes were generated per Affymetrix recommendations. Hu6800 probe arrays were then read with an argon laser in the HP GeneArray<sup>TM</sup> Scanner.

### Gene profile analysis

Raw data was normalized separately in both GeneChip and GeneSpring to maximize sensitivity and specificity by using two different mathematical algorithms. All genes identified in the lists had a twofold or greater change in expression between WT H157 and Time zero, 12 h, and 24 h time points with or without etoposide treatment were treated as replicate experiments. Only genes whose expression profiles were consistent across these time points were included in the final gene lists. Further stringency was applied by excluding genes identified as absent in both groups by GeneChip.

### Syhr green RT-PCR

RNA was isolated with RNAzol (Tel-Test). RNA samples were additionally treated with DNAse for 15 min at 37°C. PCR primers for amplification were selected utilizing Primer Express<sup>TM</sup> software (PE Applied Biosystems). All samples were normalized utilizing 18 s rRNA as an endogenous control. Each amplification was replicated in triplicate with total RNA isolated from three separate experiments. TRAIL vorsus IAPs AC Spalding et al

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