

## ORIGINAL ARTICLE

# Adenovirus-mediated IKK $\beta$ /KA expression sensitizes prostate carcinoma cells to TRAIL-induced apoptosis

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Despite the fact that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can selectively induce apoptosis in cancer cells, TRAIL resistance in cancer cells has challenged the use of TRAIL as a therapeutic agent. First, prostate carcinoma cell lines (DU145, LNCaP and PC3) were screened for sensitivity to adenovirus delivery of TRAIL (Ad5hTRAIL). As amplified I $\kappa$ B kinase (IKK) activity is responsible for the constitutive nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation leading to uncontrolled cell growth and metastasis, a dual vector approach using both an adenovirus vector (Ad) expressing the dominant-negative mutant of IKK $\beta$  (AdIKK $\beta$ /KA) and Ad5hTRAIL was employed to determine if prostate cancer cells were sensitized to TRAIL in the setting of IKK inhibition. Inhibition of the NF- $\kappa$ B pathway through IKK blockade sensitized all three prostate cancer cell lines to TRAIL, regardless of NF- $\kappa$ B activation or decoy receptor gene expression. Moreover, a novel quantitative real-time RT-PCR assay and conventional flow cytometry analysis indicated that TRAIL-resistant DU145 and LNCaP cells, but not TRAIL-sensitive PC3 cells, expressed substantial amounts of TRAIL Decoy Receptor 4. In conclusion, TRAIL decoy receptor expression appeared to be the chief determinant of TRAIL resistance encountered in prostate carcinoma cell lines.

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## Introduction

Chemotherapy and radiotherapy are among the most commonly used treatment modalities for cancer. Tumor suppressor gene p53 is required for both of these treatment methods to function as antitumor agents.<sup>1</sup> But, more than 50% of human tumors acquire p53 mutations in the course of tumorigenesis.<sup>2</sup> As a result, tumors without a functional p53 gene are resistant to both chemotherapy and radiotherapy.<sup>3</sup> However, death ligands induce apoptosis independent of the cells' p53 status.<sup>4</sup> Thus, direct induction of cell death through the activation of death receptors is a viable complementary approach to conventional treatment modalities.<sup>5,6</sup> Among the death ligands tested, tumor necrosis factor (TNF)<sup>7</sup> and FasL<sup>8</sup> have been demonstrated to efficiently induce apoptosis in cancer cells. But their systemic use in cancer gene therapy is not feasible due to their systemic toxicity. On the other hand, TNF-related apoptosis-inducing ligand (TRAIL)<sup>9</sup> is not toxic for normal cells, but selectively induces

apoptosis in cancer cells.<sup>10</sup> Despite these properties, TRAIL resistance observed in some cancer cell lines represents a handicap for any proposed gene therapy approach utilizing TRAIL as a death ligand.<sup>11</sup>

Two different hypotheses have been asserted to explain the molecular mechanisms of TRAIL resistance. The first hypothesis suggests that normal cells carry decoy receptors (TRAIL-R3, TRAIL-R4) that compete against death receptors (TRAIL-R1, TRAIL-R2) for binding to TRAIL.<sup>12</sup> These receptors either dilute TRAIL ligands (like TRAIL-R3) or supply antiapoptotic signals (like TRAIL-R4) to cells. While the presence of decoy receptor gene expression can account for TRAIL resistance in normal cells, the lack of decoy receptor gene expression in tumor cells would be expected to lead to TRAIL sensitivity.<sup>13</sup> The second hypothesis advocates the presence of apoptosis inhibitory substances, such as cFLIP (FLICE inhibitory protein), in cancer cells.<sup>14</sup> Intriguingly, chemotherapeutic agents augmented TRAIL-induced apoptosis in prostate cancer cells through upregulation of death receptors<sup>15</sup> and/or downregulation of cFLIP expression.<sup>16</sup> In addition, engagement of the TRAIL-R1 receptor, using an antibody in combination with doxorubicin, selectively killed prostate cancer cells.<sup>17</sup> Despite these studies investigating TRAIL receptors in prostate cancer, a direct link between TRAIL sensitivity and

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the pattern of TRAIL receptor gene expression is yet to be proven for prostate cancer cells.

Both TRAIL-induced and the endogenous nuclear factor- $\kappa$ B (NF- $\kappa$ B) activities present in cancer cells have recently been under heavy investigation due to NF- $\kappa$ B's role in the constitution of TRAIL resistance.<sup>18</sup> The fact that NF- $\kappa$ B-stimulating agents upregulated the synthesis of TRAIL-R3 decoy receptor,<sup>19</sup> and apoptosis inhibitors such as Bcl-xL,<sup>20</sup> cIAP<sup>21</sup> and cFLIP,<sup>22</sup> suggested that NF- $\kappa$ B activation may contribute to TRAIL resistance in at least four different ways. Interestingly, TRAIL-R4 receptor engagement stimulated the NF- $\kappa$ B signaling pathway and blocked TRAIL-induced cell death.<sup>23</sup> Furthermore, TRAIL binding to death receptors (TRAIL-R1 and TRAIL-R2) also activated the NF- $\kappa$ B pathway.<sup>24,25</sup> Thus, why some cancer cells still undergo apoptosis, despite the activation of the antiapoptotic NF- $\kappa$ B pathway, is not known.

To examine the contribution of the NF- $\kappa$ B pathway to TRAIL resistance, prostate carcinoma cell lines were analyzed in terms of basal NF- $\kappa$ B activation using NF- $\kappa$ B-mediated transcription activation assay.<sup>26</sup> Following the screening for TRAIL resistance, complementary gene therapy modalities targeting the NF- $\kappa$ B pathway through the inhibition of I $\kappa$ B kinase (IKK)<sup>27,28</sup> were also utilized to determine whether this approach is useful in breaking down TRAIL resistance in prostate cancer cell lines. Lastly, the TRAIL receptor composition of prostate cancer cells and its connection to TRAIL resistance were studied in detail using both conventional flow cytometry and novel quantitative real-time RT-PCR techniques.

## Materials and methods

### *Production of recombinant adenovirus vectors*

Recombinant adenoviral stocks Ad5hTRAIL,<sup>29</sup> AdIKK $\beta$ KA,<sup>26</sup> Ad enhanced green fluorescent protein (EGFP),<sup>27</sup> AdCMVLacZ<sup>30</sup> and AdNF $\kappa$ BLuc<sup>26</sup> were amplified as described previously,<sup>31</sup> and were stored in 10 mM Tris with 20% glycerol at  $-80^{\circ}\text{C}$ . AdIKK $\beta$ KA is a first-generation adenovirus vector expressing kinase inactive IKK $\beta$ . The expression of the dominant-negative IKK $\beta$  (IKK $\beta$ KA) generates functionally inactive IKK complexes by interacting with other IKK subunits. The particle titers of adenoviral stocks were obtained by  $A_{260}$  readings, and were in the range of  $10^{13}$  DNA particles/ml, whereas functional titers were measured by plaque titering on 293 cells and expression assays for encoded proteins. Typically, the particle/plaque-forming unit ratio was 50.

### *Transduction of prostate cancer cells with first-generation recombinant adenovirus vectors*

Prostate cancer cell lines LNCaP, PC3 and DU145 (ATCC) were grown in RPMI 1640 medium supplemented with 10% FBS, 2.2 g/l sodium bicarbonate, 1 mM L-glutamine and 1% penicillin-streptomycin mixture. All cells were maintained at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  environment. Prostate cancer cell lines were transduced

with adenoviral vectors expressing the EGFP reporter gene (AdEGFP). Briefly, prostate cancer cells were infected with an increasing multiplicity of infection (MOI) of AdEGFP vectors at  $37^{\circ}\text{C}$  in RPMI 1640 without FBS. The serum concentration in the tissue culture media was increased to 10% by adding an equal volume of RPMI 1640 supplemented with 20% FBS 2 h after the infection. The level of transduction was determined by assessing percent GFP (+) cells 48 h after the infection under fluorescent microscopy then using flow cytometry. Cell viability was determined by propidium iodide (PI) exclusion. The Ad5hTRAIL construct was used to overexpress hTRAIL in prostate cancer cells. To block IKK activity, and thereby NF- $\kappa$ B activation, an adenoviral vector encoding the IKK $\beta$  dominant-negative mutant (AdIKK $\beta$ KA) was utilized in coinfection experiments in conjunction with Ad5hTRAIL. An AdNF $\kappa$ BLuc construct carrying NF- $\kappa$ B regulatory sites hooked up to a Luciferase reporter gene was deployed to conduct NF- $\kappa$ B-mediated transcription activation assays. This construct possessed a Luciferase reporter gene hooked up to the herpes simplex virus thymidine kinase gene promoter with four tandem copies of the NF- $\kappa$ B-binding consensus sequence.<sup>27</sup>

### *NF- $\kappa$ B-mediated transcription activation assay*

AdNF $\kappa$ BLuc was employed to provide information on the prostate cancer cell's NF- $\kappa$ B activation status. The Luciferase assay system with Reporter Lysis Buffer (Promega, Inc.) was used to measure NF- $\kappa$ B-mediated transcriptional induction in the presence or absence of TRAIL expression according to the manufacturer's protocol. All measurements of Luciferase activity (relative light units) were normalized to the protein concentration.

### *Assessment of cell viability*

Live/dead Cellular Viability/Cytotoxicity Kit from Molecular Probes (Eugene, OR) was used to discriminate the live cells from the dead cells. In this assay, Calsein AM, a fluorogenic substrate for intracellular calsein esterase, is modified to a green fluorescent compound (calsein), which is demonstrable only in live cells. Since only live cells with intact membranes contain active esterase, detection of calsein by fluorometric methods serves as a marker for viable cells. Ethidium homodimer-1 (EthD-1) is a red fluorescent nucleic acid stain that cannot disseminate across unharmed cell membranes. While intact cells exclude EthD-1, cells with damaged membranes take up the dye and stain positive.

### *Regular and quantitative real-time RT-PCR for human TRAIL receptors*

Total RNA was extracted from LNCaP, DU-145 and PC-3 human prostate cancer cell lines using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. In all, 2  $\mu\text{g}$  of total RNA was reverse-transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems Cat. N8080234). Regular RT-PCR reactions were carried out as described previously.<sup>14</sup> Our group has recently

published the sequences of TRAIL-R1 and TRAIL-R2 primers and probes in an article on neuroblastoma.<sup>32</sup> However, since TRAIL-R3 and TRAIL-R4 decoy receptor probes have not been published, we designed new probe sets for the TRAIL decoy receptors. The sequence of TRAIL decoy receptor sets is as follows: **TRAILR3** – 5' CCC-TAA-AGT-TCG-TCG-TCG-TCA-T, **TRAILR3** – 3' GGG-CAG-TGG-TGG-CAG-AGT-A, **TRAILR3 Probe** – 5' 6FAM-TCGCGGTCTGCTGCCAGTCCTAGC-TAM-RA 3'; **TRAILR4** – 5' ACA-GAG-GCG-CAG-CCT-CAA, **TRAILR4** – 3' ACG-GGT-TAC-AGG-CTC-CAG-TAT-ATT, **TRAILR4 Probe** – 5' 6FAM-AGGAGGAGTGTC CAGCAGGATCTCATAGATC-TAMRA 3'. The rRNA probes were labeled with a second dye to analyze TRAIL receptors and rRNA as an internal control in the same reaction. The rRNA primers and probes were purchased from PE Applied Biosystems (Cat. 4308329). A cloned cDNA fragment derived from ribosomal RNA was used to construct a standard curve. Relative quantities of TRAIL receptors were calculated using the  $\Delta\Delta Ct$  method as described by Applied Biosystems. The TaqMan PCR reaction was carried out as described in the manufacturer's protocols (Applied Biosystems Cat. N8080228).

#### Annexin V binding

An FITC-conjugated mouse monoclonal antibody to human Annexin V (ALX-804-100F-T100) was employed for Annexin V binding using flow cytometry. Annexin V binding assay was performed according to the manufacturer's instructions (Alexis Biochemicals).

#### Flow cytometry analysis for adenovirus transduction and the detection of surface TRAIL receptor expression

Prostate cancer cells were seeded at approximately  $2.5 \times 10^5$  cells per well in 24-well plates and then infected with AdEGFP reporter construct. At 48 h postinfection, the cells were trypsinized and resuspended in PBS, following centrifugation. FACS analysis was carried out using BD FACSCALIBUR at the Akdeniz University Hospitals. To assess TRAIL receptor protein expression on the cell surface, unlabeled monoclonal antibodies specific for each TRAIL receptor subtype were employed using the anti-TRAIL receptor flow cytometry set (Cat. ALX-850-273-KI01) from Alexis Biochemicals. The set contained 100  $\mu\text{g}$  each of MAbs to TRAIL-R1 (clone HS101, Cat. 804-297A), -R2 (clone HS201, Cat. 804-298A), -R3 (clone HS301, Cat. 804-344A) and -R4 (clone HS402, Cat. 804-299A). All primary antibodies were used at 5  $\mu\text{g}/\text{ml}$  concentration, followed by biotinylated goat anti-mouse IgG1 (Cat. ALX-211-202) and streptavidin-PE (Cat. ANC-253-050). Flow analysis was performed according to manufacturer's protocols. Purified mouse IgG1 (MOPC 31C, Cat. ANC-278-010) was used as an isotype control.

#### Statistical analysis

The Prism program from GraphPad Software (San Diego, CA) was used for statistical analyses. The statistical

results for each experiment are provided in the figure legends.

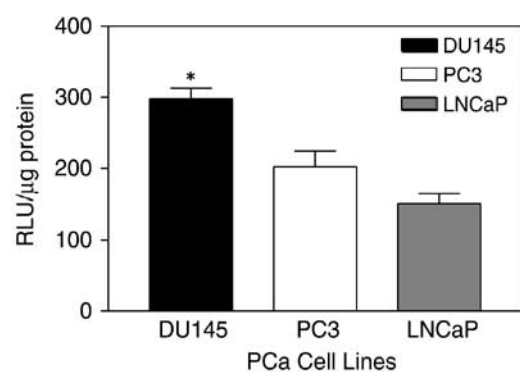
## Results

### Prostate carcinoma cell lines exhibited differential basal NF- $\kappa$ B activation levels

As constitutive NF- $\kappa$ B activation is claimed to be a prominent factor influencing the sensitivity of tumor cells to the apoptosis-inducing effects of death ligands,<sup>28,33–35</sup> unstimulated (basal) NF- $\kappa$ B activation levels were analyzed using three different prostate cancer cell lines (DU145, PC3 and LNCaP). Prostate cancer cell lines were infected with a recombinant adenovirus vector carrying the NF- $\kappa$ B-driven Luciferase reporter gene (AdNF $\kappa$ BLuc) for 24 h prior to harvesting. NF- $\kappa$ B-mediated transcription activation assays were performed using the Luciferase assay system with reporter lysis buffer. As shown in Figure 1, the highest constitutive NF- $\kappa$ B activation level was detected in DU145 cells, followed by PC3. The lowest NF- $\kappa$ B activation was observed in LNCaP cells. Luciferase assays conducted 48 h following the infection yielded a much higher magnitude of NF- $\kappa$ B activation, but this did not change the order of activation, DU145 leading PC3, followed by LNCaP (data not shown). AdCMVLacZ infection did not generate any readable Luciferase activity compared to uninfected controls (data not shown). Thus, the prostate cancer cell lines PC3, LNCaP and DU145 exhibited substantially different levels of constitutive NF- $\kappa$ B activation.

### Differences in constitutive NF- $\kappa$ B activation levels detected in prostate cancer cells were not caused by differential adenovirus transduction

A reporter adenovirus vector encoding the enhanced green fluorescent gene (AdEGFP) was infected into



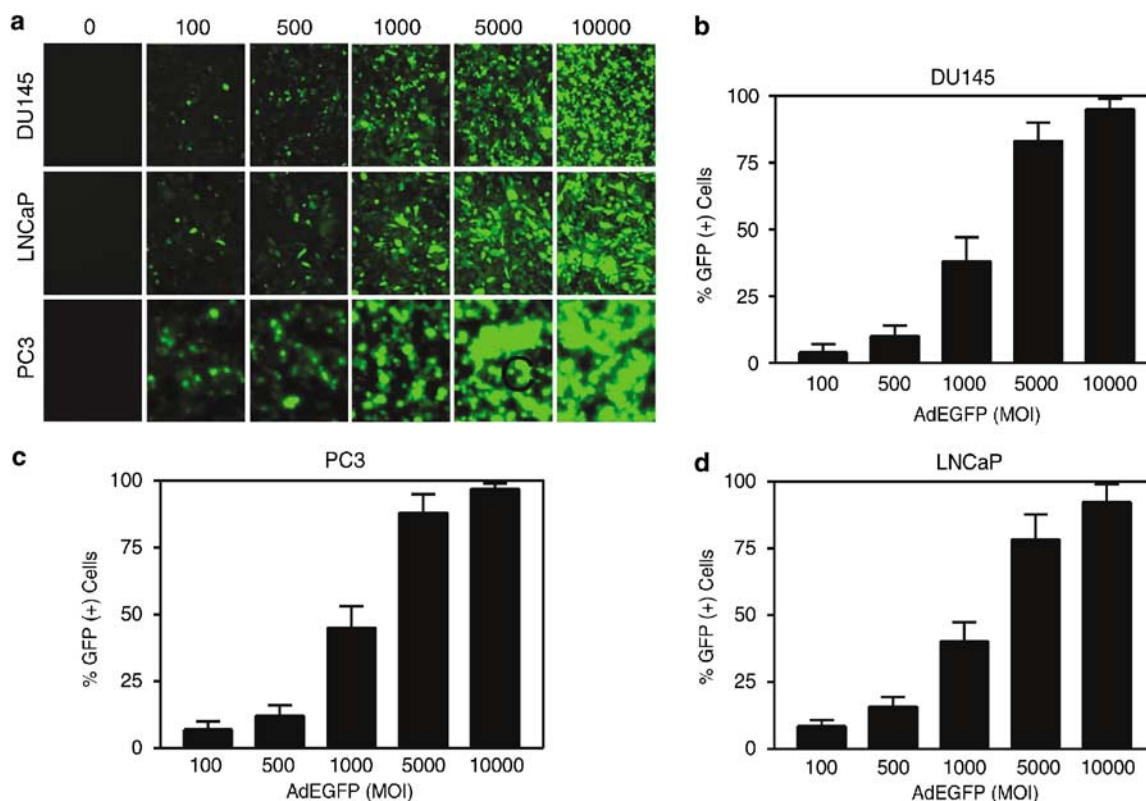
**Figure 1** Prostate cancer cell lines display diverse NF- $\kappa$ B activation levels. Prostate cancer cell lines were infected with an MOI of 5000 DNA particles/cell of AdNF $\kappa$ BLuc construct for 24 h. Luciferase activity was measured as described in Materials and methods. Cell types used in the infection are provided on the x-axis. Luciferase activity expressed as relative light units (RLU) per microgram protein is given on the y-axis. Data represent the mean ( $\pm$ s.e.m.) of six independent data points. ANOVA followed by Tukey's multiple comparison tests indicated the existence of a statistically significant difference between DU145 versus PC3 and LNCaP. \* $P < 0.01$ .

prostate cancer cells at increasing MOIs to determine if differences in NF- $\kappa$ B activation among the prostate carcinoma cell lines were due to differential transduction by adenovirus vectors. Percent EGFP-positive cells were determined by fluorescent microscopy (Figure 2a) and quantified by flow cytometry (Figure 2b–d) 48 h following the infection. Flow cytometry indicated that equal levels of adenoviral transduction were observed in all three prostate carcinoma cell lines. These results clearly demonstrated that differences in basal NF- $\kappa$ B activity were not due to differential adenovirus transduction of prostate carcinoma cell lines. This experiment was also pivotal in determining the optimum adenoviral transduction dose of prostate carcinoma cell lines for gene therapy purposes. While an MOI of 5000 DNA particles/cell of AdEGFP was sufficient to transduce more than 90% of prostate carcinoma cell lines, almost 100% transduction efficiency was achieved when cells were infected with an MOI of 10 000 DNA particles/cell. Nonetheless, all three prostate carcinoma cell lines were transduced efficiently and equally by AdEGFP. Thus, the differential transduction by adenovirus cannot be accounted for by the disparity in NF- $\kappa$ B activation observed in prostate carcinoma cell lines.

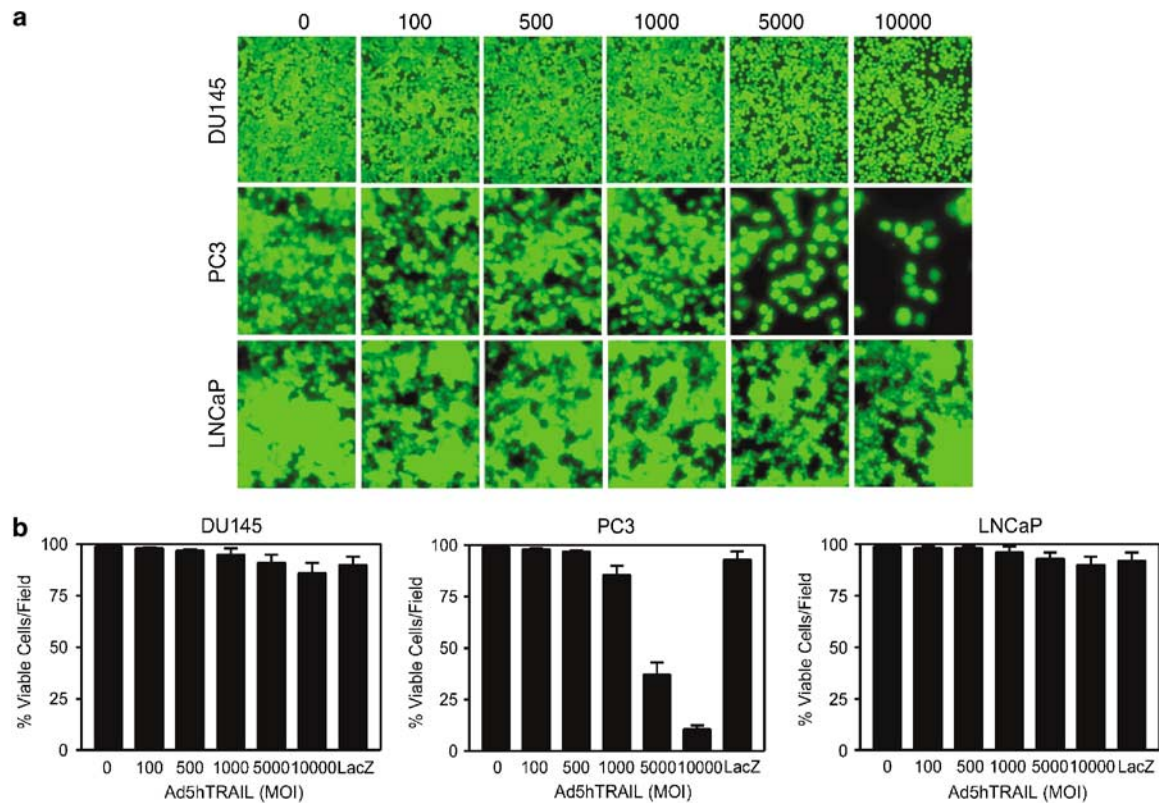
### Prostate carcinoma cell lines manifested diverse levels of TRAIL resistance

The cytotoxic effects of TRAIL overexpression in prostate cancer cell lines were examined by infecting the cells with increasing doses of Ad5hTRAIL or AdCMVLacZ vectors. Cell viability assays were conducted using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit 48 h following the infection (Figure 3a). Analysis under fluorescent microscopy revealed that prostate carcinoma cell lines LNCaP and DU145 were highly resistant to TRAIL, even when these cells were infected with an MOI of 10 000 DNA particles/cell of Ad5hTRAIL virus. No significant cell death was observed upon AdCMVLacZ infection (Figure 3b). On the other hand, PC3 cells exhibited 63% cell death at an MOI of 5000 DNA particles/cell and 90% cell death at an MOI of 10 000 DNA particles/cell of Ad5hTRAIL virus (Figure 3b). Thus, the PC3 cell line showed substantially greater levels of TRAIL sensitivity than the DU145 or LNCaP cell lines.

Recently, elevated NF- $\kappa$ B activation in prostate carcinoma cell lines has been attributed to increased IKK activity.<sup>36</sup> Therefore, we sought to determine whether inhibition of IKK activity might decrease the viability of



**Figure 2** Transduction of prostate carcinoma cell lines by first-generation recombinant adenovirus vectors. Prostate carcinoma cell lines were infected with an adenovirus encoding the EGFP reporter gene (AdEGFP) at increasing MOIs for 48 h as described in Materials and methods. Panel a displays fluorescent micrographs of such adenovirus transductions. MOI values (DNA particles/cell) of viruses used in the infection are given above each fluorescent micrograph. Corresponding results from flow cytometry are illustrated in panel b (DU145), panel c (PC3) and panel d (LNCaP). Numbers displayed on the x-axis represent viral doses applied in MOI values as DNA particles/cell. Values represent the mean ( $\pm$ s.e.m.) of three different experiments.



**Figure 3** Prostate cancer cells exhibit distinctive patterns of TRAIL sensitivity. LNCaP, DU-145 and PC3 cells were infected with increasing MOIs of Ad5hTRAIL or AdCMVLacZ constructs. Cell viability was detected using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit 48 h following the infection. Only fluorescent micrographs of FITC channel are shown in panel a. Numbers represent viral doses applied in MOI values as DNA particles/cell. Quantitative results from the cell viability assays of prostate carcinoma cell lines are provided in panel b. LacZ columns in panel b refer to MOIs of 10 000 DNA particles/cell of AdCMVLacZ vector used in the infection. Cell viability assays were performed in triplicates and repeated at least twice to confirm the observation. Values represent the mean ( $\pm$ s.e.m.) of six independent data points.

prostate cancer cells. An adenovirus expressing the dominant-negative mutant of IKK $\beta$  (AdIKK $\beta$ KA) was infected into prostate cancer cell lines at increasing MOIs. Cell viability was examined under the fluorescent microscope 48 h following the infection. Contrary to the TRAIL cytotoxicity observed in PC3 cells, no significant decrease in cell viability, even at an MOI of 10 000 DNA particles/cell of AdIKK $\beta$ KA construct, was observed in any of the three prostate carcinoma cell lines (data not shown). Therefore, infection with IKK inhibiting adenovirus vector alone did not induce cell death in prostate cancer cell lines 48 h following infection.

*While TRAIL overexpression increased intracellular NF- $\kappa$ B activity, IKK inhibiting strategy counteracted both TRAIL-induced and endogenous NF- $\kappa$ B activity* TRAIL decoy receptor TRAIL-R4<sup>23</sup> and TRAIL death receptors (TRAIL-R1 and TRAIL-R2)<sup>24,25</sup> have been shown to activate the NF- $\kappa$ B pathway. If TRAIL is to be used as a death ligand to induce apoptosis in cancer cells, then TRAIL-induced NF- $\kappa$ B activation and the cell's endogenous NF- $\kappa$ B status should be considered before the therapy. To study the extent of NF- $\kappa$ B activation by TRAIL expression in prostate carcinoma cell lines, PC3,

LNCaP and DU145 cells were coinfecting with Ad-NF $\kappa$ BLuc and Ad5hTRAIL vectors. To minimize cell death, the Ad5hTRAIL concentration was kept constant at an MOI of 1000 DNA particles/cell and NF- $\kappa$ B Luciferase assays were conducted 24 h following the infection. As shown in Figure 4, NF- $\kappa$ B activity was increased only in TRAIL-overexpressing prostate cancer cells.

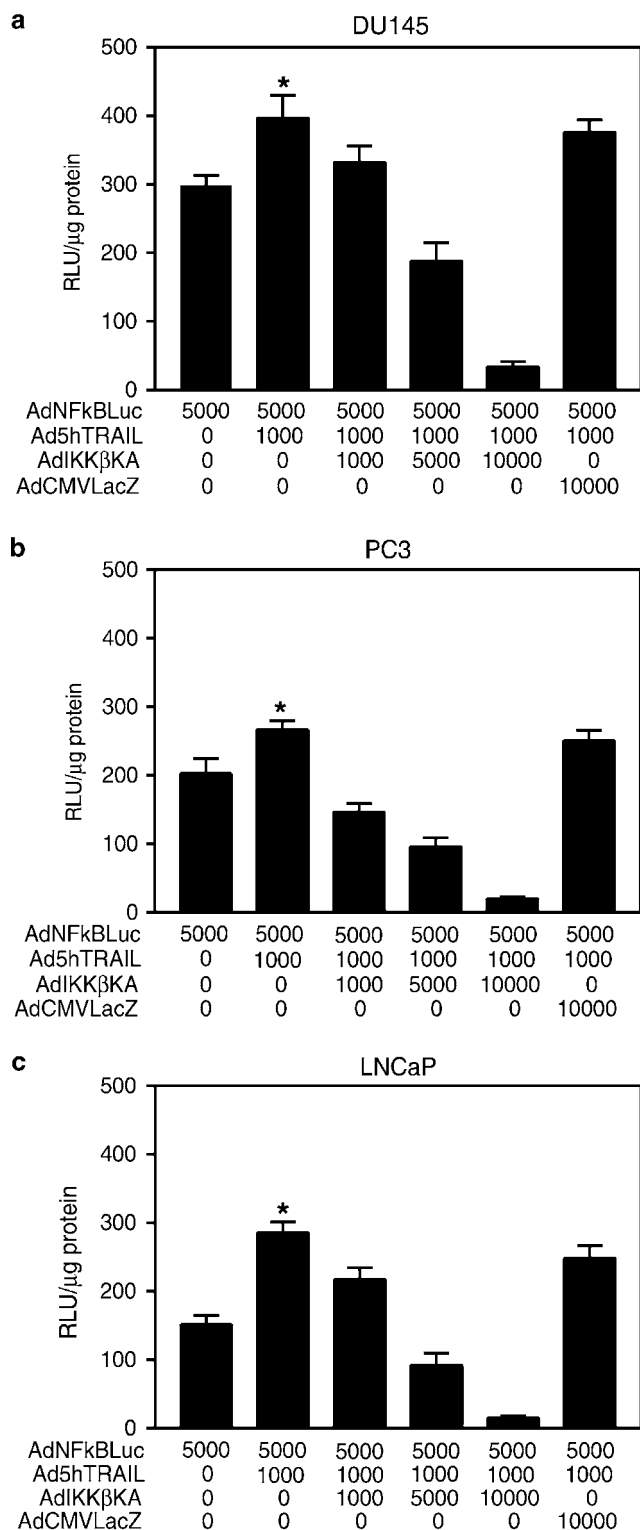
To determine the magnitude of NF- $\kappa$ B inhibition, prostate carcinoma cell lines were superinfected with AdIKK $\beta$ KA virus at increasing doses in addition to coinfection with AdNF $\kappa$ BLuc and Ad5hTRAIL. TRAIL-induced NF- $\kappa$ B activity and basal NF- $\kappa$ B activities were drastically reduced in all three prostate carcinoma cell lines (Figure 4a–c). In contrast, no such NF- $\kappa$ B inhibiting effect was observed when cells were superinfected with AdCMVLacZ virus as a control.

#### *Functional IKK inhibition via IKK $\beta$ KA expression-sensitized prostate cancer cells to TRAIL-induced cell death*

NF- $\kappa$ B-inhibiting strategies involving the use of adenovirus delivery of IKK $\beta$  (AdIKK $\beta$ KA)<sup>27,35</sup> or IkB $\alpha$ (AdIkB $\alpha$ SR)<sup>30,37</sup> dominant-negative mutants have

been successful in sensitizing lung cancer cells to TNF death ligand. As some cancer cells have higher intrinsic NF- $\kappa$ B activity, NF- $\kappa$ B-blocking agents can potentially be very valuable to sensitize these cells to the apoptosis-inducing effects of TRAIL. To test whether IKK

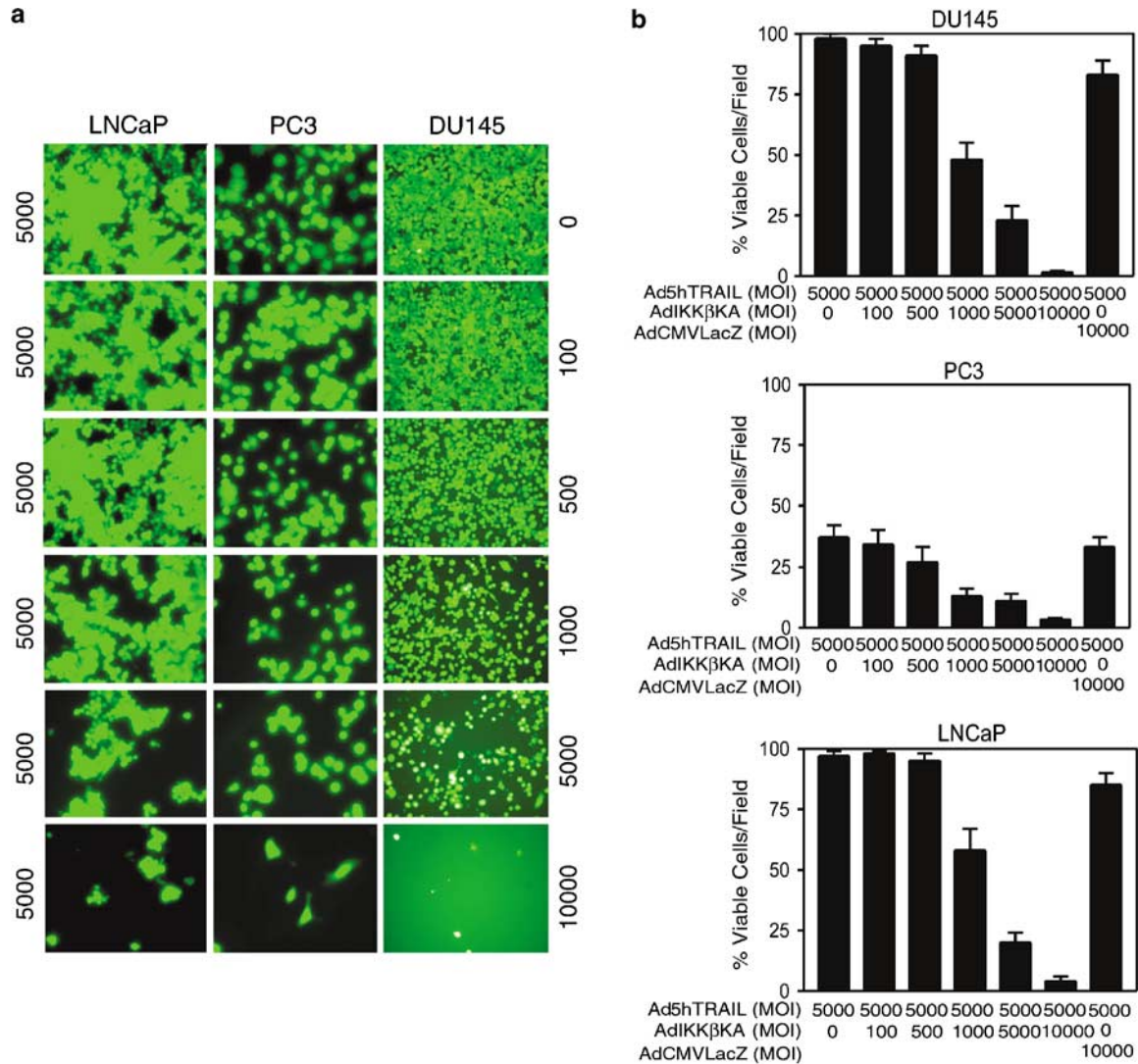
inhibition sensitizes prostate carcinoma cell lines to TRAIL, the cell lines were coinfecting with both Ad5h-TRAIL construct at a constant MOI of 5000 and increasing doses of AdIKK $\beta$ KA virus. All three prostate carcinoma cell lines were sensitized to TRAIL regardless of the cell's prior constitutive NF- $\kappa$ B activation status (Figure 5a). Approximately 50% cell death was observed, even at an MOI of 1000 AdIKK $\beta$ KA virus in the presence of TRAIL expression in DU145 and LNCaP cell lines. As PC3 cells were already sensitive to TRAIL, the degree of TRAIL sensitization induced by IKK $\beta$ KA expression in DU145 and LNCaP cells were much higher than in PC3 cells (Figure 5b). On the other hand, no TRAIL sensitization was observed when prostate carcinoma cell lines were infected with AdCMVLacZ virus in place of AdIKK $\beta$ KA. Therefore, NF- $\kappa$ B blocking through IKK inhibition might be very useful in the treatment of prostate cancer patients displaying TRAIL resistance.



*Ad5hTRAIL and AdIKK $\beta$ KA coinfection-induced apoptotic cell death in prostate carcinoma cells*

Annexin V staining was performed using flow cytometry to reveal the molecular mechanism of cell death induced by TRAIL under the setting of IKK inhibition. For this purpose, Ad5hTRAIL and AdIKK $\beta$ KA vectors were infected separately or in combination into the prostate carcinoma cell line DU145. Annexin V binding assays were conducted 35 h following the infection using flow cytometry. As shown in Figure 6a, Ad5hTRAIL or AdIKK $\beta$ KA infection alone did not generate any significant degree of Annexin V binding. However, when the cells were coinfecting with both Ad5hTRAIL and AdIKK $\beta$ KA, considerable levels of Annexin V binding were observed, indicating that prostate carcinoma cells were undergoing apoptosis (Figure 6b). On the other hand, AdCMVLacZ coinfection (negative control) together with Ad5hTRAIL did not yield any substantial levels of Annexin V binding, suggesting that, in the absence of IKK inhibition, DU145 cells were resistant to TRAIL expression. Taken together, these results suggest that the cell death induced by TRAIL in the setting of IKK inhibition is apoptosis.

**Figure 4** NF- $\kappa$ B activity of prostate cancer cell lines is increased by Ad5hTRAIL infection, but downregulated by AdIKK $\beta$ KA. DU145 (a), PC3 (b) and LNCaP (c) cell lines were simultaneously infected with AdNFκBLuc, Ad5hTRAIL and/or increasing doses of AdIKK $\beta$ KA constructs for 24 h. These cell lines were also infected with AdCMVLacZ as a control. Cells were harvested for luciferase activity 24 h after the infection. Both the MOI values provided as DNA particles/cell and the types of constructs used in the infection are given on the x-axis. To avoid cell death complicating our assay result, the titer of Ad5hTRAIL was lowered to an MOI of 1000 DNA particles/cell instead of 5000 or 10000 used in cell viability assays. The y-axis shows the luciferase activity expressed in RLU per microgram protein. Data represent the mean ( $\pm$ s.e.m.) of six independent data points. Student's *t*-test was used to reveal the statistical difference between AdNFκBLuc and AdNFκBLuc/Ad5hTRAIL coinfecting cells. \**P*<0.05.



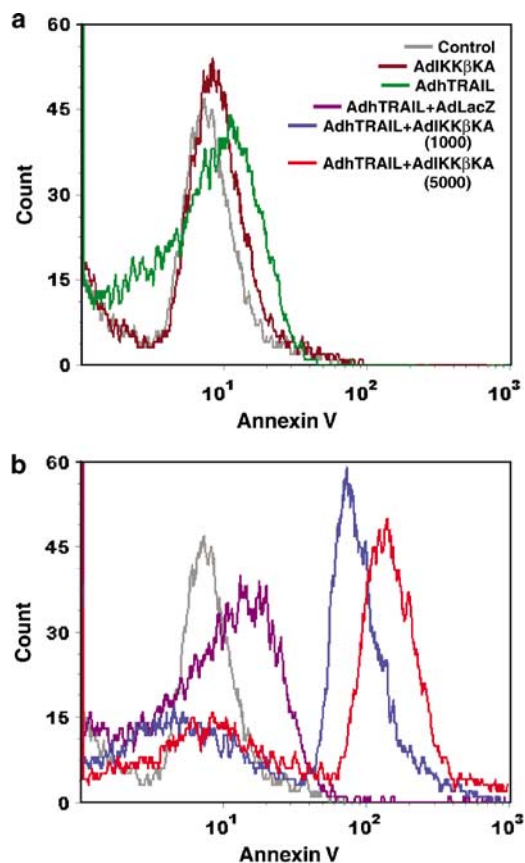
**Figure 5** IKK $\beta$ KA expression via adenoviral vectors defeated the resistance to TRAIL-induced apoptosis in prostate carcinoma cell lines. Adenoviral vectors encoding the dominant-negative mutant of IKK $\beta$  or LacZ were infected into LNCaP, DU145 and PC3 cells at increasing doses (shown to the right of fluorescent micrographs), while simultaneous infection with Ad5hTRAIL was performed at a constant MOI of 5000 DNA particles/cell (as shown to the left of fluorescent micrographs). Cell viability was assessed using Live/Dead Cellular Viability/Cytotoxicity Kit from Molecular Probes 48 h following infection. Fluorescent micrographs of cell viability assays (only FITC channel) are provided in panel a. Corresponding percent viable cell counts/ $\times 20$  field are shown in panel b for DU145, PC3 and LNCaP cells. Numbers represent viral doses applied in MOI values of DNA particles/cell as depicted on the x-axis. Values represent the mean ( $\pm$ s.e.m.) of six independent data points.

*Differential TRAIL receptor expression patterns were observed in prostate cancer cell lines*

Although RT-PCR is a quick and useful test in assessing whether a relevant gene is expressed in a particular cell line, the technique does not provide quantitative information regarding gene expression. For this reason, a quantitative real-time RT-PCR analysis was conducted using primer-probe sets specifically designed to detect TRAIL receptor gene expression in prostate carcinoma cell lines. As shown in Figure 7, levels of TRAIL-R2 gene expression were higher than levels of TRAIL-R1 in all the cell lines. PC3 cells, which are TRAIL-sensitive, expressed greater levels of the TRAIL-R2 receptor than did the

DU145 and LNCaP cell lines, which are TRAIL-resistant (Figure 7). While very low levels of TRAIL-R3 expression were detectable in PC3 cells, no TRAIL-R4 expression was found. Furthermore, TRAIL-resistant DU145 and LNCaP cell lines expressed both decoy receptors (TRAIL-R3 and TRAIL-R4) at significant levels.

While real-time RT-PCR is useful in quantifying gene expression at the mRNA level, gene expression inside the cell does not necessarily correlate with the receptor expression on the cell surface. Therefore, we decided to analyze the level and type of TRAIL receptor expression on the cell surface using flow cytometry. Although PC3 cells expressed TRAIL death receptors (TRAIL-R1 and

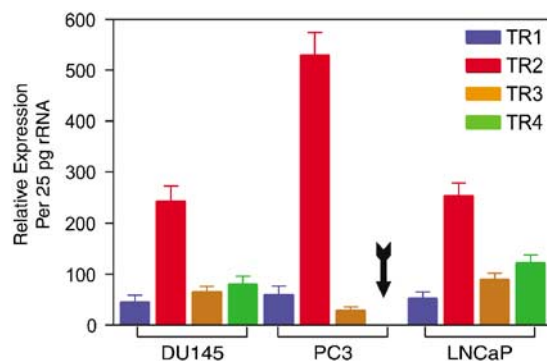


**Figure 6** TRAIL and IKK $\beta$ KA expression induce apoptosis in DU145 prostate cancer cell line. Infections with Ad5hTRAIL and AdIKK $\beta$ KA or AdLacZ (negative control) were performed as described in Materials and methods. All virus constructs were used at an MOI of 5000 DNA particles/cell unless stated otherwise in the figure. Infected DU145 cells were stained with both FITC-conjugated Annexin V and PI, prior to flow analysis. Uninfected, but FITC-Annexin V- and PI-stained, cells served as controls. Each histogram represents  $10^4$  gated DU145 cells. Histograms were illustrated in two panels for clarity. Treatment conditions were depicted in panel a. The Annexin V binding assay was repeated independently three times to confirm the observation, and only one such representative assay was provided in the figure.

TRAIL-R2) on the cell surface, no measurable level of decoy receptor gene expression was evident on the surface of these cells (Figure 8b). Despite the fact that DU145 (Figure 8a) and LNCaP cells (Figure 8c) expressed both types of TRAIL death receptors, contrary to PC3, there were substantial amounts of TRAIL-R4 decoy receptors on the surface of both cell types. Furthermore, some degree of surface TRAIL-R3 decoy receptor expression was detectable in LNCaP cells, but not in DU145 or PC3 cells.

## Discussion

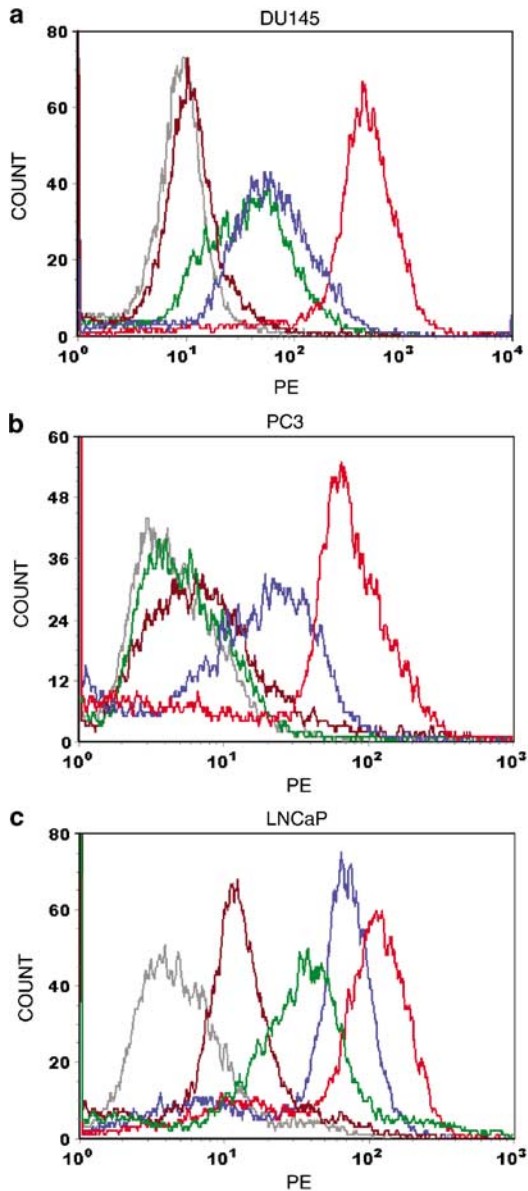
Prostate cancer is one of the leading causes of cancer death in North American men. As advanced prostate



**Figure 7** Prostate carcinoma cell lines display distinctive TRAIL receptor expression patterns. RT-PCR assays were performed using total RNA isolated from LNCaP, DU145 and PC3 cells to determine the level of expression (quantitative real-time) of TRAIL receptors. A cloned ribosomal cDNA fragment was used to generate a standard curve for relative expression. TRAIL receptor levels per 25 pg of ribosomal cDNA are presented in the graph. Ribosomal RNA primers and probes were included in each TaqMan assay as an internal control. Arrow indicates the absence of TRAIL-R4 decoy receptor expression in PC3 cells.

carcinoma is highly resistant to conventional treatments, adenovirus delivery of death ligands is a viable complementary gene therapy approach.<sup>28</sup> Despite the promise of TRAIL death ligand for the treatment of prostate cancer, recent studies have demonstrated that some prostate cancer cell lines are TRAIL-resistant, and this resistance has been attributed to the constitutively active AKT kinase,<sup>11</sup> a key regulator of NF- $\kappa$ B transcription factor.<sup>38,39</sup> Demarchi *et al* have demonstrated that AKT-induced NF- $\kappa$ B activation requires IKK activity.<sup>40</sup> Since increased IKK activity results in the constitutive NF- $\kappa$ B activation observed in prostate carcinoma cell lines,<sup>36</sup> we hypothesized that coinfection of prostate carcinoma cell lines with adenovirus vectors expressing the dominant-negative mutant form of IKK $\beta$  (AdIKK $\beta$ KA) and functional hTRAIL (Ad5hTRAIL) would sensitize advanced prostate cancer cells to TRAIL.

Three different prostate carcinoma cell lines (DU145, PC3 and LNCaP) were used to test this hypothesis. As a first step, basal NF- $\kappa$ B activation levels in prostate cancer cell lines were revealed by NF- $\kappa$ B-mediated transcription activation assays using an adenovirus carrying NF- $\kappa$ B regulatory sites fused to a Luciferase reporter gene (Figure 1). In our experimental conditions, DU145 exhibited the highest levels of NF- $\kappa$ B activity, followed by PC3 and LNCaP cells. The differences in basal NF- $\kappa$ B activation levels were not due to the differential transduction of prostate cancer cells by adenovirus vectors (Figure 2). Next we screened three prostate cancer cell lines for TRAIL resistance. Contrary to some previous reports,<sup>41</sup> cellular viability/toxicity assays indicated that DU145 and LNCaP cells were resistant to Ad5hTRAIL-induced apoptosis. Furthermore, in contrast to a report from Beresford and coworkers,<sup>42</sup> PC3 cells exhibited considerable levels of TRAIL sensitivity (Figure 3). While some discrepancies can be attributed to the form of



**Figure 8** Cell surface expression patterns of TRAIL receptors in prostate carcinoma cell lines. Surface TRAIL receptor expressions of DU145 (a), PC3 (b) and LNCaP (c) cells were detected using monoclonal antibodies specific for each TRAIL receptor according to the manufacturer's protocol using flow cytometry. Colored lines indicate experimental parameters. Gray: isotype-specific control, blue: TRAIL-R1, red: TRAIL-R2, maroon: TRAIL-R3, green: TRAIL-R4.  $10^4$  cells were gated for each histogram. Each assay was repeated three times to confirm the results. Only one representative assay for each experiment is shown.

TRAIL used for functional studies (purified soluble form versus viral delivery), other inconsistencies might be due to the functional titer of the virus used in infection. Recently, several studies have been conducted to overcome TRAIL resistance in cancer cells. For instance, ionizing radiation<sup>43</sup> and chemotherapeutic agents<sup>44</sup> have sensitized cancer cells to TRAIL through upregulation of TRAIL death receptors. As increased IKK activity was

blamed for the constitutive NF- $\kappa$ B activation responsible for the survival of prostate carcinoma cell lines,<sup>36</sup> we sought to sensitize advanced prostate carcinoma cell lines to TRAIL using a complementary gene therapy modality involving IKK inhibition. As shown in Figure 5, TRAIL-resistant DU145 and LNCaP prostate cancer cell lines were sensitized to TRAIL only when cells were coinfecting with AdIKK $\beta$ KA virus. To rule out possibilities for TRAIL resistance other than NF- $\kappa$ B, we analyzed the pattern of TRAIL receptor gene expression in prostate carcinoma cell lines.

Previously, the use of regular RT-PCR assays to screen human tumor cell lines, such as melanoma, colon carcinoma, breast adenocarcinoma and lung adenocarcinoma, did not reveal any connection between TRAIL resistance and TRAIL receptor gene expression.<sup>10</sup> Despite this fact, we wanted to confirm whether or not the expression pattern of TRAIL receptors is connected to TRAIL resistance in prostate cancer cells. A quantitative real-time RT-PCR assay was conducted using specific probe sets for each TRAIL receptor (Figure 7). Substantial levels of TRAIL-R3 and TRAIL-R4 decoy receptor gene expressions were detected only in TRAIL-resistant DU145 and LNCaP cells. Why PC3 cells do not express TRAIL-R4 is yet to be determined. Intriguingly, aberrant promoter methylation leading to the silencing of TRAIL decoy receptors (TRAIL-R3 and TRAIL-R4) was recently confirmed in multiple tumor types, including prostate cancer (60%).<sup>45</sup> As mRNA levels do not necessarily correlate with protein expression on the cell surface, flow cytometry was employed to determine the level of TRAIL receptor protein on the cell surface (Figure 8). Considerable levels of TRAIL-R4 decoy receptor protein expression were detectable only in the TRAIL-resistant DU145 (Figure 8a) and LNCaP cells (Figure 8c), but not in TRAIL-sensitive PC3 cells (Figure 8b). In addition, unlike the case for DU145 or PC3 cells, surface TRAIL-R3 decoy receptor was expressed on LNCaP cells (Figure 8c). Considering the fact that the absence of death receptors also led to TRAIL resistance as shown recently for rheumatoid arthritis synovial fibroblasts,<sup>46</sup> we have concluded that the presence of decoy receptors, but not the lack of death receptors, correlates with TRAIL resistance in prostate carcinoma. These results are supported by studies indicating that TRAIL-sensitive target cells transfected with either TRAIL-R3 or TRAIL-R4 decoy receptors display a reduction in apoptotic cell death.<sup>12,47</sup> Intriguingly, the TRAIL-R4 decoy receptor was more effective in protecting cells from TRAIL-mediated apoptosis than was TRAIL-R3.<sup>12</sup>

These results demonstrate that both the TRAIL decoy receptor composition and the cell's intracellular NF- $\kappa$ B activity are two major players contributing to TRAIL resistance in prostate cancer cell lines. In addition, IKK inhibiting strategies overwhelmed TRAIL resistance. For this reason, the adenovirus-mediated TRAIL gene delivery approach under the setting of IKK inhibition should be valuable in expanding the therapeutic index of TRAIL for patients with prostate carcinoma.

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