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# Detachment-mediated resistance to TRAIL-induced apoptosis is associated with stimulation of the PI3K/Akt pathway in fetal and adenocarcinoma epithelial colon cells

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The resistance of transformed epithelial cells to a detachment-induced apoptosis (anoikis) can significantly affect their susceptibility to anticancer therapy. We showed that detachment of both fetal (FHC) and adenocarcinoma (HT-29) human colon epithelial cells resulted in the activation of the pro-survival Akt pathway, and significant changes in integrin-linked kinase (ILK) and focal adhesive kinase (FAK) phosphorylation. We demonstrated a detachment-induced and PI3K/Akt-mediated resistance to apoptotic effects of TRAIL, which was not associated with any changes in the cell surface TRAIL death receptor levels. Instead, a modulation of downstream intracellular signaling events was suggested to be involved. Our results may have important implications for optimization of new strategies in treatment of cancers at different stages of development.



## 1. Introduction

The interactions between epithelial cells and extracellular matrix (ECM) are essential for tissue organization in colonic crypts, cell adhesion, differentiation, proliferation, survival, migration, or re-attachment. Normal epithelial cells at the top of the crypt lose contacts with ECM and neighboring cells, and die by anoikis, a detachment-induced apoptosis. Resistance of transformed epithelial cells to anoikis promotes their invasion and metastasis, and the resulting anchorage-independent growth is a crucial step in tumorigenesis [1]. Therefore, understanding and targeting defects in the signaling pathways responsible for anoikis resistance is crucial for the development of efficient anticancer strategies.

Integrin-linked kinase (ILK) and focal adhesion kinase (FAK) belong to key enzymes involved in integrin-mediated signal transduction and focal adhesion (FA) complex formation. FAK was shown to be overexpressed in tumors, which correlated with increased cancer cell motility, invasiveness, survival, and proliferation [2]. FAK-deficient cells spread more slowly on ECM proteins and migrate poorly in response to chemotactic signals. ILK is implicated in regulation of anoikis [3] and its expression can serve as important prognostic marker in patients suffering from different forms of cancer including colon. Mutations of the specific ILK phosphorylation sites are associated with reduced cell motility, and proliferation [4]. Moreover, it was recently shown that ILK interacts with both initiator caspase-8 and -9 in an adhesion-dependent manner [5].

PI3K/Akt pathway mediates majority of proliferative signals in intestinal epithelial cells both *in vitro* and *in vivo*, and often suppresses apoptosis or anoikis [6]. Various factors can be responsible for stimulation of this pathway, including those elicited by FAK or ILK [7]. Promotion of cell survival by the activation of this pathway often occurs by the inhibition of proapoptotic and/or the induction of pro-survival molecules [8,9], e.g. antiapoptotic Mcl-1 and cFLIP proteins [10,11], both of them being shown to play an important role in anoikis regulation [12].

TRAIL, (TNF)-related apoptosis inducing ligand, is known for its unique properties to induce death of cancer cells (including colon) while sparing most normal cells [13]. TRAIL ligation of death receptors DR4 and/or DR5 results in formation of the death-inducing signaling complex (DISC), and pro-caspase-8 processing, which can be efficiently regulated by cFLIP [14,15]. Caspase-8 activation leads to the stimulation of two distinct apoptotic pathways, dependent or independent on mitochondria [16]. TRAIL-induced apoptosis can be modulated by Bcl-2 family proteins [17], FAK [18] or PI3K/Akt pathway [19]. The resulting signaling can finally significantly affect the type of response to TRAIL in attached versus detached epithelial cells.

We compared changes in sensitivity/resistance to TRAIL-induced apoptosis in adherently and non-adherently cultivated epithelial cell lines derived from human colon fetal or adenocarcinoma tissue,

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with a particular focus on the role of selected kinases and antiapoptotic proteins. Here we report on detachment-mediated fetal (FHC) and adenocarcinoma (HT-29) colon epithelial cell resistance to TRAIL-induced apoptosis and its association with stimulation of the PI3K/Akt pathway.

## 2. Material and methods

### 2.1. Cell lines and reagents

The FHC cell line derived from normal human fetal colon epithelium (ATCC, Rockville, MD, USA) was cultured in DMEM/F12 medium (Sigma Aldrich, Munich, Germany) supplemented with HEPES (25 mM), cholera toxin (10 ng/ml; Calbiochem-Novabiochem Corp.; La Jolla, CA, USA), insulin (5 μg/ml), transferrin (5 μg/ml), and hydrocortisone (100 ng/ml; all Sigma Aldrich). Human colon adenocarcinoma HT-29 cells (ATCC) were cultured in McCoy's 5A medium (Sigma Aldrich) supplemented with gentamycin (50 mg/l; Sigma Aldrich). Both cell lines were grown with 10% fetal bovine serum (FBS; PAN Systems, Aidenbach, Germany) at 37 °C in 5% $CO_2$ and 95% humidity, and were passaged twice a week after exposure to trypsin/EDTA (0.05/0.02%). 24 h after seeding (adherent cultivation) or immediately after seeding (non-adherent cultivation), the cells were treated with TRAIL (human Killer TRAIL, 100 ng/ml, provided by L. Andera) for the times indicated.

The PI3K/Akt pathway was inhibited by InSolution™ Akt Inhibitor VIII (Calbiochem-Novabiochem, 0.5 μM) or wortmanin (WRT, Axxora, LLC, San Diego, CA, USA, 100 nM). PF573228 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 40 μM) was used as FAK kinase inhibitor.

### 2.2. Model of non-adherent cultivation

We established an experimental model of non-adherent cultivation based on slow continuous culture media movement using a 3D rotator (Omni-Bio, Brno, Czech Republic). Under these conditions, the trypsinized cells were unable to adhere to culture surface, and had a tendency to form aggregates in suspension [20].

### 2.3. Apoptotic nuclear morphology (fluorescence microscopy)

The cells were stained with a 4,6-diamidino-2-phenyl-indole (DAPI, Fluka, Buche, Switzerland) solution (1 μg DAPI/ml ethanol) at room temperature in the dark for 30 min. They were then mounted in Mowiol 4-88 (Calbiochem-Novabiochem) and the percentage of apoptotic cells (with chromatin condensation and fragmentation) was determined using a fluorescence microscope (Olympus 1×70, Olympus, Prague, Czech Republic) from a total number of 200 cells.

### 2.4. Western blot analysis

Cells were harvested and protein extracts were subjected to electrophoresis and western bloting as described in Hofmanova et al. [21]. The membranes were probed with mouse anti-caspase-3 (#7272), rabbit anti-phosphoILK (#130196), rabbit anti-PARP (#7150) (Santa Cruz Biotechnology), rabbit anti-Akt (#9272), rabbit anti-phosphoAkt (#9271), rabbit anti-ILK (#3856), rabbit anti-cFLIP (#3210), mouse anti-caspase-8 (#9746) (Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit anti-DR4 (D3813), rabbit anti-Mcl-1 (M8434), mouse anti-β-actin (A5441) (Sigma–Aldrich), mouse anti-FAK (#610087), mouse anti-phosphoFAK (#611722) (BD Biosciences, NJ, USA), and goat anti-DR5 (#210-743, Axxora) antibodies. The proteins recognized were detected using horseradish peroxidase-labeled secondary antibodies: mouse anti-IgG (1:3000, #NA931), rabbit anti-IgG (1:6000, #NA934) (Amersham Biosciences, Bucks., UK), goat anti-IgG (1:4000, A4174, Sigma Aldrich), and an enhanced chemiluminescence kit (ECL, Amersham Biosciences or Immobilon Western HRP Substrate, Millipore Corp.). An equal loading was verified using β-actin quantification.

### 2.5. Expression of TRAIL death receptors

The cells were washed with PBS, and incubated 45 min at 4 °C with FITC-conjugated mouse DR4 (Axxora, Anti-TRAIL-R1, DR4 HS101 FITC, 1:100) or DR5 (Axxora, Anti-TRAIL-R2, DR5 HS201 FITC, 1:100) primary antibodies. After another washing, 7-AAD (1 μg/ml) was added. Expression of surface DR4 and DR5 was analysed using flow cytometry (BD Biosciences FACSCalibur) in cells negative for 7-AAD fluorescence. Cell surface expression was expressed as a ratio of the median fluorescence index (MFI) of the specific antibody and the isotype control antibody.

### 2.6. Statistical analysis

The results of at least three independent experiments were expressed as the means ± SD. Statistical significance ($P < 0.05$) was determined by one-way ANOVA followed by a Tukey test or a non-parametric Mann–Whitney test.

## 3. Results

### 3.1. Detachment of HT-29 and FHC cells resulted in their increased resistance to TRAIL-induced apoptosis

During adherent cultivation, a significant increase in the occurrence of morphologically detected signs of apoptosis (fragmenta-

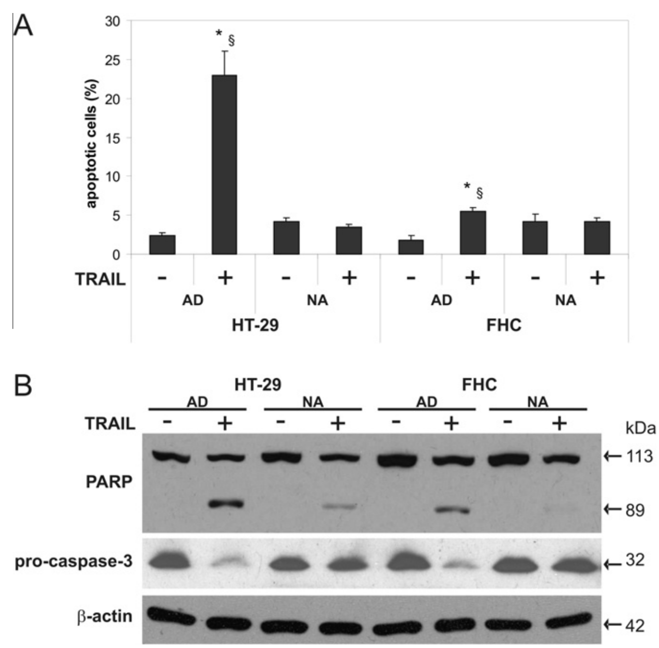


**Fig. 1.** HT-29 and FHC cells with (A) apoptotic nuclear morphology (DAPI staining, fluorescence microscopy) and (B) cleavage of PARP, and pro-caspase-3 (Western blotting) after treatment (24 h) with TRAIL (100 ng/ml) during adherent [AD] and non-adherent [NA] cultivation. The data in (A) are presented as mean ± SD from three independent experiments. In (B) β-actin was used as loading control and similar results were obtained in three independent experiments. $P < 0.05$, (*) versus appropriate control; (§) adherent versus non-adherent cultivation.