Ras sensitizes colon cells to apoptosis by TRAIL

TRANSFORMATION BY ONCOGENIC RAS SENSITIZES HUMAN COLON CELLS TO TRAIL INDUCED APOPTOSIS BY UPREGULATING DR4 AND DR5 THROUGH A MEK-DEPENDENT PATHWAY

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Ras oncogenes play a major role in cancer development by activating an array of signaling pathways, most notably Mitogen Activated Protein Kinases (MAPKs) resulting in aberrant proliferation and inhibition of apoptotic signaling cascades, rendering transformed cells resistant to extrinsic death stimuli. However, Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL), is able to kill specifically tumor cells through the engagement of its receptors Death Receptor 4 (DR4) and Death Receptor 5 (DR5) and the activation of apoptotic pathways, providing promising targets for anticancer therapies. In this study, we show that TRAIL induces cell death in human colon adenocarcinoma cells in a MEK-dependent manner. We also report a prolonged MEK dependent activation of ERK1/2 and increased c-fos expression induced by TRAIL in this system. Our study reveals that transformation of the colon cell line Caco-2 by Kirsten- and mainly by Harvey-ras oncogenes sensitizes these cells to TRAIL induced apoptosis by causing specific MEK-dependent upregulation of DR4 and DR5. These observations taken together reveal that Ras-MEK-ERK1/2 signaling pathway can sensitize cells to TRAIL induced apoptosis, by upregulating DR4 and DR5 and overall imply that TRAIL based therapeutic strategies using TRAIL agonists could be used in cases of human colon cancers bearing ras mutations.

The initiating mutagenic changes in the genome producing an early adenoma from normal colonic mucosa may provide a favorable environment (i.e. increased replication rate, increased survival signals, alterations of the DNA repair mechanisms) for other mutations to occur. In this way, they traverse, from early to intermediate to late adenoma and finally adopt a malignant phenotype, as characterized by the multistage carcinogenesis model (1). Avoidance of apoptosis is a basic event that occurs during carcinogenesis. Changes in the apoptotic program and/or activation of the antiapoptotic pathways are key events allowing progression through various stages of carcinogenesis (2).

Apo2L/TRAIL (Tumor Necrosis Factor Related Apoptosis Inducing Ligand / Apo2 Ligand) is a cytokine that can selectively induce apoptosis on tumor cells while leaving normal cells largely unaffected. The effects of TRAIL are mediated through the TNF receptor superfamily members, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). TRAIL can also bind to the decoy receptors, whose role remains to be identified (3, 4). There are 4 distinct TRAIL receptors, all belonging to the TNF receptor superfamly: TRAIL-R1 (DR4) (5), TRAIL-R2 (DR5) (6), TRAIL-R3 (TRID/DcR1/LIT) and TRAIL-R4 (DcR2) (7). The latter two are incapable of transducing the signal as they either do not contain a death domain or contain a truncated death
Ras sensitizes colon cells to apoptosis by TRAIL domain. TRAIL ligation to its functional receptors leads to recruitment of the adapter protein Fas-associated death domain (FADD) to the cytoplasmic region of the receptor (8) followed by recruitment of procaspase 8 or procaspase 10 (9, 10) resulting in the formation of the Death Inducing Signalling Complex (DISC). At this level, pro-caspases undergo autocalytic processing to be activated leading to the activation of the effector caspasases (11), mitochondrial dysfunction and ultimately apoptosis.

Even though TRAIL receptor activation is mainly linked to the induction of apoptosis, evidence implicate TRAIL signalling in the activation of JNK (12), NFkB (13, 14) and ERK (15, 16), suggesting that multiple signaling pathways are activated through the TRAIL/TRAIL-R system. It has been shown that incubation with TRAIL can activate a positive feedback loop causing the upregulation of TRAIL-R2 (17) that can potentially render the cells more sensitive to TRAIL in a time dependent manner. Activation of AP-1 (18) can lead to TRAIL-R1 upregulation. Similarly TRAIL-R2 has been shown to be upregulated through NFkB (17). These results taken together allow us to assume that molecules not directly implicated to the TRAIL pathway play an important role in the regulation of TRAIL receptor expression.

Considering the limitations of the current therapies in targeting cancer cells only, TRAIL provides an attractive target for cancer therapy (19). Currently preclinical studies using soluble recombinant TRAIL derivatives as well as monoclonal antibodies mimicking TRAIL effects induce apoptosis in a broad range of human cancer cells and tumor regression in mice without significant systemic toxicity (4). In addition, it can complement current strategies as it can render death sensitivity to tumors by its synergistic action with chemotherapeutic drugs or UV-treatment (20, 21).

Ras genes play a very important role in the development of human cancer. Activating mutations in the three best studied ras genes, Ha-ras, Ki-ras and N-ras, have been detected in a large variety of human tumors (24). Studies in mice that are genetically modified to express ras oncogenes have very clearly shown that activated Ras promotes the initiation, progression and maintenance of several types of tumors (25, 26). Activating Ki-ras mutations are found in about 50% of colon carcinomas. Activated Ras mediates its biological activity through interaction with various downstream effector targets, thus activating pathways like MEK, PI3K and Rho GTPases. Ras regulates a Raf-MEK-ERK1/2 kinase cascade. The MEK pathway has been shown to be active in human colon adenocarcinoma cells (27) as well as in human colorectal tumors (28). The activated ERK1/2 translocate to the nucleus and regulate the expression of target genes like c-fos and/or fra-1 (29, 30).

Sensitization to TRAIL induced apoptosis very often is mediated by upregulation of TRAIL receptors DR4 and DR5 (22). Interestingly, expression of DR4 and DR5 has been found elevated in human colonic carcinomas as compared to normal colonic mucosa (23), we aimed to pinpoint oncogenic pathways that make TRAIL more efficient against cells representing the latter stages of carcinogenesis. We specifically examined the sensitivity of an intermediate colon adenocarcinoma cell line, Caco-2, to TRAIL induced apoptosis subsequent to its transformation with two active Ras isoforms (KRasV12 and HRasV12), which are very potent activators of ERK1/2 kinases. Caco-2 cells have previously been reported to be insensitive to TRAIL induced apoptosis. We found that in Caco-2 human colon cells, the two ras oncogenes present tumor promoting potential, the Ha-ras clones giving more and larger tumors in nude mice and a different gene expression profile (Roberts et al under revision). In this study we provide strong evidence for the importance of ERK in the regulation of TRAIL-R1 and TRAIL-R2. Moreover, we denote the significance of ras oncogenes in the regulation of TRAIL receptors and the subsequent sensitivity to TRAIL mediated apoptosis, both via cellular transformation and through direct signaling.

Specifically, we demonstrate the functional importance of the ERK-1/2 signal in modulating TRAIL-induced apoptosis in Ras-transformed cells, where aberrant Ras activation upregulates the expression of TRAIL receptors, thus modulating the subsequent sensitivity to TRAIL mediated apoptosis by an ERK-dependent mechanism. We also demonstrate the relevance of this mechanism to its potential exploitation in colorectal cancer therapy.
Materials and Methods

**Cell Cultures**— The Caco-2, DLD1 and HT29 were obtained from ATCC. Human colon cell lines, Caco-2, HT-29 and the ras overexpressing clones derived from Caco-2 were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), antibiotics and non-essential aminoacids (Gibco). Human colon adenocarcinoma cell line, DLD-1 and the derived clone which has the oncogenic Ki-ras allele disrupted (DKO-4) (40) were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco) and antibiotics. Human colon cell lines, AAC1 and RGC2 obtained from Prof. C. Paraskeva, UK were cultured in the same manner as the HT-29 cells with the addition of insulin, hydrocortisone and 20% fetal bovine serum (Gibco). RGC2 and AAC1 are clonogenic, non-tumourigenic human colonic adenoma-derived cell lines (41). MEK inhibitors PD 98059 and U0126 were purchased from Alexis Biochemicals. PI3-K inhibitor Wortmannin was purchased from Sigma. The Caco-2 clones constitutively expressing active Ras proteins have already been described (Roberts et al, under revision). In short, cDNA expressing either V12 Ki-Ras4B or Ha-RasV12 was ligated into pcDNA3 plasmid. The resulting plasmid was transfected into Caco-2 cells using the CaPO4 precipitation technique and selected with Geneticin (Gibco). The colonies expressing between two and three fold of exogenous protein were selected for further analysis. As a control, Caco-2 cells were also transfected with empty pcDNA3 expression cassette and a number of colonies were isolated and expanded.

**FACS analysis**— For immunostaining, 5x10^5 cells were pre-incubated with blocking buffer (PBS containing 0.2% gelatin, 0.1% sodium azide and 20% FBS) and then incubated in the staining buffer (PBS containing 0.2% gelatin and 0.1% sodium azide) with mAb (50 µg/ml) on ice for 30 min. After washing, cells were incubated in the staining buffer containing PE-conjugated GAM (#1070-09 – Southern Biotechnology Associates). Cells were then washed, resuspended in the staining buffer, and analyzed by flow cytometry. The antibodies used against the following antigens: DR4 - Alexis HS101, DR5 - Alexis HS201, DcR1 - Alexis HS301, DcR2 - Alexis HS402, Fas- Exbio LT95, CD29 -Exbio MEM101A, IgG1- GAM-PE (#1070-09 – Southern Biotechnology Associates). At least 4000 viable (negative propidium iodide staining) cells were analyzed for each condition.

**RT-PCR**— RNA isolation was performed using the TRIZOL Reagent (Life Technologies). cDNA was prepared using an oligo(dT) primer and Moloney leukemia virus reverse transcriptase (Promega) following standard protocols. Primers used in this experiments were: TRAIL-R1, 5_- CAGAACGTCTGGAGCCTGTAAC- 3_ and 5_- ATGTCCATTGGCCTGATTTTGTG-3; TRAIL-R2, 5_- GGGAAAGAAGATCTCCTGAGATGTG-3_ and 5_- ACATTGTCTCCAGCCCCAGGTGC-3; GAPDH, 5_- ACCACAGTCCATGCCATCAC-3_ and 5_- TCCACCACCTGTTGCTGTA-3; c-Fos, 5_- GAATAAGATGGCTGCAGCCAAATGC-3_ and 5_- AAGGAAGACGTGTAAGCAGTGCAGC-3. After reverse transcription the cDNA product was amplified by PCR with 3 units of Taq DNA polymerase (Promega) and 2.5mM Mg2+ using standard protocols; the annealing temperature was 56°C for all primers except for the c-fos primers (58°C). The amplified products were separated on 1.2% agarose gels, stained with ethidium bromide, and photographed using ultraviolet illumination. Intensity values were measured using Molecular Dynamics ImageQuant Software (Amersham Biosciences). All PCR products were normalized to GAPDH expression.

**Cytotoxicity and Apoptosis Assays**— For cell viability cells were plated on 6-well plates, fixed with absolute methanol, stained with 0.5% crystal violet for 10min, washed 3 times with PBS and the remaining crystal violet was extracted using 30% acetic acid, absorbance was measured at 595nm. The percentages of viable, necrotic, and apoptotic cells were assessed by exposure to the DNA binding dyes Hoechst No. 33342 (Sigma) and propidium iodide (Sigma), apoptotic measurements were carried out using a fluorometric method and counting cell numbers under a fluorescent inverted microscope (Nikon Eclipse).

**Immunoblotting** — Whole cell lysates prepared with the following lysis buffer: 50mM Tris.HCl pH 7.4, 250 mM Sucrose, 1 mM EDTA, 10mM NaF, 1mM EGTA and 1% Triton X-100 supplemented with protease and phosphatase inhibitors and the lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Immunoblotting was performed with the following
Ras sensitizes colon cells to apoptosis by TRAIL antibodies at 1:1000 (v/v): Caspase-8 (Santa Cruz Biotechnology Inc., sc-6134), Caspase-3 (Santa Cruz Biotechnology Inc., sc-1225), PARP (Santa Cruz Biotechnology Inc., sc-8007), p-ERK1/2 (Santa Cruz Biotechnology Inc., sc-7383), ERK-2 (Santa Cruz Biotechnology Inc., sc-1647), p-MEK1/2 (Cell Signaling, #9121S), Flip (Alexis, ALX-804-428), TRAIL-R1 (ProSci, No. 1139), TRAIL-R2 (ProSci, No. 2019), FADD (BD Transduction Laboratories, No. 610399), p-Akt-Ser473 (Cell Signaling, #9271S). The membranes were scanned with Image Storm Scanner (Molecular Dynamics) and the values were measured using Molecular Dynamics ImageQuant Software (Amersham Biosciences).

ERK2 protein expression and/or Ponceau staining was used for protein loading control; ERK2 was also used for normalization. All experiments were repeated at least 3 times and standard deviation function was used for error bar generation. All controls in every experiment were treated with the same amount of buffer/dilutant as in the treated cells.

RESULTS

TRAIL induces apoptosis specifically in colon carcinoma cell lines and this effect correlates with high DR4 and DR5 expression levels. TRAIL has been shown to exert enhanced apoptotic activity on tumor cells while non-tumor cells have been reported to be resistant to TRAIL induced death in many systems (4). In order to examine the apoptotic effects of TRAIL in an in vitro system of human colorectal carcinogenesis, we subjected human adenoma and carcinoma colon cells to different concentrations and time points of TRAIL treatment.

The three colon adenoma cell lines examined in the study, AAC1 (41), RGC2, and Caco-2 showed no difference in viability after 24h treatment with 500ng/ml TRAIL (Fig. 1A). Even after 72h of TRAIL treatment no evidence of cell death in adenoma cells was detected (data not shown). On the other hand, the carcinoma cell lines HT-29 and DLD-1 had reduced viability after TRAIL treatment (Fig.1A) by undergoing apoptosis as detected by Hoechst staining and PARP cleavage; a representative image of the HT29 cell line is shown. (Fig.1B). Similar results were obtained with the DLD-1 cell line (data not shown). The expression levels of the functional TRAIL receptors and its downstream signaling components were examined both at the mRNA level by RT-PCR (Fig. 1C) and at the protein level by WB analysis (Fig.1D), respectively. We detected increased levels of DR4 and DR5 mRNA and protein levels in HT29 and DLD-1 cells as compared to Caco-2 cells that were correlated to the sensitivity of the cell lines to TRAIL induced cell death. Expression levels of other factors like FADD, caspase 8 and caspase 3 did not correlate with sensitivity of the examined cell lines to TRAIL induced apoptosis (Fig. 1D).

TRAIL induces a rapid and sustained MEK dependent activation of ERK1/2 followed by high mRNA levels of c-fos. TRAIL receptor activation has been reported to regulate MAP kinase signalling pathways like JNK (12) and ERK (15), and these pathways play an important role in TRAIL induced apoptosis in many systems. To examine the cascade of MAP kinase signaling events in the different colon cell lines after TRAIL treatment, we have followed the activation of MEK pathway from the cell membrane to the nucleus. Steady state basal phosphorylation levels of the ERK1/2 kinases were detected in colon cell lines (Fig. 2A). Treatment with TRAIL increased the phosphorylated levels of MEK and p42/44 (ERK1/2) in HT29 cells (Fig. 2A). There was a strong and rapid ERK1/2 activation peaking 15 min after treatment with 500ng/ml TRAIL. ERK phosphorylation followed MEK activation that peaked after approximately 5 min of treatment with TRAIL. We then examined the Elk-1-responsive immediate early gene, c-fos, in the HT29 cells, and found an increase at the mRNA level of 1.9 fold at 30 min and 2.5 fold at 90min after treatment with TRAIL, while this increase was abolished by co-treatment with the MEK inhibitor PD98059 (Fig. 2B). Remarkably, c-fos induction by TRAIL was sustained for 90min and did not show the characteristic transient induction observed in many systems after growth factor stimulation (Fig. 2B). The DLD-1 cell line was also found to have increased mRNA levels of the c-fos gene after 25min of incubation with TRAIL, effect that was abolished by co-treatment with the MEK inhibitor (data not shown).

Treatment with TRAIL increases the levels of its receptors, DR4 and DR5, while MEK inhibition reduces TRAIL’s ability to induce apoptosis by...
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downregulating their expression. Incubation with TRAIL has been reported to activate a positive feedback loop causing the upregulation of TRAIL-R2 (DR5) (17) and this effect contributes in sensitizing cells to TRAIL induced cell death in these cases, thus showing the importance of the regulation of TRAIL signaling at the receptor level. To explore the mechanism of TRAIL induced MEK signaling and its effect on TRAIL receptor expression, we examined the potential feedback mechanism in the colon cell lines.

Pre-treatment of the HT29 cell line for 16h with 100µM MEK inhibitor PD98059 prevented TRAIL from significantly reducing cell viability (Fig. 3A) and from inducing apoptosis. Specifically, HT29 cells treated for 24h with TRAIL showed significant reduction in viability relative to the control. Pretreatment for 16h with the MEK inhibitor of cells to be treated with TRAIL resulted in increase of cell viability (Fig. 3A), inhibition of PARP cleavage (Fig. 3B), a reduced number of apoptotic nuclei (Fig. 3C) and reduced caspase 8 activation (data not shown). Specifically, 32% (± 3.8) of the nuclei counted in the cells treated with TRAIL had apoptotic characteristics while in the case of cells pretreated with the MEK inhibitor PD98059 only 16% (± 2.1) of the nuclei showed apoptotic characteristics after treatment with TRAIL (Fig. 3C). On the other hand, in the DLD-1 cell line MEK inhibition failed to produce any significant differences in TRAIL induced cell death (data not shown).

Additional evidence for the regulatory role of MEK pathway on the expression levels of TRAIL receptors was provided by the reduced steady state levels of the functional TRAIL receptors DR4 and DR5 after treatment with MEK inhibitor, as determined by RT-PCR in the DLD-1 and HT29 cells (Fig. 4A) and Western Blot analysis in the HT29 cells (Fig. 4B). This clearly showed that in both the HT29 and the DLD-1 cell lines the expression levels of these receptors were, at least, partially dependent on MEK activation. This effect was lower on the DLD-1 cells indicating that in this cell line the role of MEK signaling in the expression of the Death Receptors is not very important. In the same experiment we have shown that the mRNA expression levels of both TRAIL receptors increases after treatment with TRAIL (Fig. 4A). To determine if the actual expression levels of the DR4 and DR5 receptors were altered on the cell surface, FACS analysis was performed on the HT29 cells confirming the observations made by Western Blot and RT-PCR. There was a slight decrease in cell surface levels of DR4 and DR5 after 1h incubation with MEK inhibitor PD98059 while 16h incubation showed approximately 42% (± 4.2) and 51,5% (± 4.6) decrease in the levels of DR4 and DR5 respectively. The decoy and Fas receptors showed no significant changes after treatment with PD98059, indicating that the effect of the MEK inhibitor was not universal for death receptors levels in the cells under investigation (Fig. 5).

Human colon cells transformation by oncogenic Ki- and Ha-ras upregulates DR4 and DR5 followed by increased sensitivity to TRAIL. To examine the ability of Ras oncogenes, which are very potent activators of ERK1/2 kinases, to sensitize non transformed human cells to TRAIL induced apoptosis, we examined if their overexpression would sensitize the previously unresponsive intermediate colon adenoma cell line Caco-2, to TRAIL induced apoptosis.

The Caco-2 cell line was stably transfected with the Ki-rasV12, Ha-rasV12 and the empty vector (Neo) as a control (Roberts et al under revision) and clones with low-moderate expression of ras were chosen for analysis. The response of the various ras transformed cells to TRAIL was determined by cell viability assay and the presence of PARP cleavage. The Ha-ras transformed cells (H2 and H13) showed responsiveness to TRAIL in reducing cell viability by approximately 35% after 24h and by 73% after 62h treatment while the Ki-ras (K15) clones were more resistant, beginning to show significantly reduced viability after 62h of treatment (Fig. 6A); these clones give representative results of the various Neo, Ki- and Ha-ras transformed cells tested. The reduced viability occurred by apoptosis as detected by Hoechst staining (data not shown) and by Western Blot for the characteristic to apoptosis PARP cleavage (Fig. 6B). Moreover, we examined the steady state levels of phosphorylated Akt at Ser473, a downstream effector of PI-3K survival signaling, to see if these levels correlate with the differential responsiveness of Ki- and Ha-ras transformed cells to TRAIL. Steady state levels of p-Akt were higher in both clones relative to neo although Ha-ras seemed to be more effective in activating PI-3K signaling (Fig. 6C) as previously reported in other systems (42). Using a PI-3K
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inhibitor (wortmannin) we were able to reduce the phosphorylation levels of Akt in all clones (Fig. 6C) with a corresponding reduction in viability after TRAIL treatment (Fig. 6D). These results show a uniform response of the cells to PI-3K inhibition allowing us to assume that it is unlikely that PI-3K signaling plays an important role in the differential responsiveness of the clones to TRAIL.

In order to determine the expression levels of DR4 and DR5 in the ras overexpressing clones, we performed RT-PCR and Western Blot analysis. RT-PCR analysis of the steady state mRNA levels for DR4 and DR5 indicated their significant overexpression in the ras transformed clones in both cases as compared to the control (Neo) transfected cells (Fig. 7A). Western Blot analysis of the expression levels of DR4 and DR5 showed an upregulation of these receptors of an 1.7 fold average while caspase 3, caspase 8 and FADD protein levels did not show significant differences between the ras clones; remarkably in the case of DR5 protein levels in the Ha-ras clones there is an additional slower migrating band repeatedly giving a strong signal on Western Blot at about 50KDa (Fig 7B). Overexpression of DR4 and DR5 in the Caco2 ras transformed cells were confirmed and on the actual expression on the cell surface by FACS analysis (Fig. 7C). In addition to DR4 and DR5 upregulation, we noted that in the case of the Ha-ras clones there is a strong upregulation and cell surface localization of the Fas receptor as analyzed by FACS (Fig. 7C) while in both cases the decoy receptor levels were not altered as compared to the control Caco-2 neo clones. To further confirm the effect of oncogenic ras on DR4 and DR5 expression we checked the steady state mRNA expression of these receptors in DKO-4 cells, which were derived from DLD-1 cells and have their oncogenic Ki-ras allele disrupted (40). RT-PCR analysis showed that both DR4 and DR5 expression is reduced in the DKO-4 clone relative to the DLD-1 cells transfected with the control cassette (Fig. 7D).

**Inhibition of ERK1/2 in the Ki- and Ha-ras clones partially reverses the increased expression levels of DR4 and DR5.** We investigated whether ras transformation of Caco-2 cells was followed by a corresponding increased ERK activity; WB analysis for activated ERK1/2 showed increased phosphorylation levels of ERK1/2 in both the Ki- and the Ha-ras clones that could be decreased after MEK inhibition (Fig. 8A). The use of the specific inhibitors for MEK reduced the increased expression of DR4 and DR5 on the mRNA level (Fig. 8B) as well as the protein level as detected by Western Blot analysis (Fig. 8C). The decrease in protein and mRNA levels of DR4 and DR5 was accompanied by a respective decrease in sensitivity to TRAIL induced apoptosis when the H2 clone was treated with MEK inhibitor PD98059 prior to treatment with TRAIL (Fig. 8D). We note that these results were reproducible when we used the U0126 compound for specific inhibition of MEK (data not shown).

**DISCUSSION**

The discovery of specific cellular targets for colorectal cancer therapy resulting in the generation of selective agents against aberrantly regulated gene products present in cancer cells will potentially result in less toxicity observed in traditional therapies. As such, the most attractive therapeutic agent targets regulated cell properties like survival, cell cycle, signal transduction and metastasis. Using this approach, studies are currently in progress in order to predict which targets and therapeutic candidates will be most promising in the treatment of colorectal cancer (33).

Constitutively activated Ras increases the tumorigenic potential of cells as it causes deregulation of important intracellular signaling pathways. Ras proteins transduce signals from receptor tyrosine kinases to a downstream cascade of protein kinases regulating the growth, survival and cytoskeletal processes that are aberrant in malignant cells. Activating mutations of ras occur in almost half of colorectal cancer incidence. Major Ras regulated signaling pathways are the MEK and PI3K pathways, which play an important role in cell proliferation and survival.

The death receptor ligands tumor necrosis factor (TNF), Fas ligand (FasL) and TRAIL are all able to induce apoptosis by binding to their cell membrane receptors. Recombinant forms of these ligands can potentiate the antitumor effects of cytotoxic agents in both in vitro and in vivo models.
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TRAIL induced cell death in colon cells is correlated with DR4 and DR5 overexpression in a MEK dependent manner

We have used human colon cell lines to show that the apoptotic agent TRAIL activates the MEK pathway in a rapid and sustained manner, and that this pathway is involved in regulating DR4 and DR5 expression levels. Specifically, by inhibiting MEK we found that the mRNA and protein levels of DR4 and DR5 were downregulated and this had a striking effect on the ability of TRAIL to induce cell death in the HT29 cells and the oncogenic ras transformed Caco-2 clones. Moreover, we found that in cells non-responsive to TRAIL-induced apoptosis ERK1/2 activation by overexpression of activated RasV12 isoforms consequently upregulated expression of DR4 and DR5 and sensitized the cells to death while disruption of oncogenic Ki-ras reduced both the phosphorylation levels of ERK proteins and Death Receptors expression. Our findings are further supported by the evidence that expression of the human death receptor 4 is regulated by AP-1 (18). On the other hand transcriptional upregulation of the death receptor 5 gene is dependent on NF?B (17) and AP-1 (18). Therefore, TRAIL receptors expression as well as sensitivity of cells to TRAIL, besides MEK, is regulated by multiple factors including signaling pathways, transcription factors and the general oncogenic transformation status of the cells due to co-operative activities of existing oncogenic mutations. We believe that in the case of the DLD-1 cells these factors have a high impact on their sensitivity. It has to be noted that DLD-1 cells bear a Ki-ras as compared to B-Raf mutation in HT-29 cells, as well as a different APC mutation than HT-29 cells, which result in differential signaling to the nucleus (43). These taken together with the observation that these cells constitutively express much higher levels of TRAIL receptors and, therefore, after treatment with PD98059 still conserve high levels of these receptors can explain the inability of MEK inhibition to reduce their sensitivity.

Our findings provide a mechanistic explanation of how MEK signals promoting cell growth in most cases, can also under certain conditions mediate cell death, in this case by inducing elevated expression of TRAIL receptors and sensitizing resistant cells to TRAIL induced cell death. The decision between life and death in a particular cell type must be dependent on the balance of signaling pathways and in the case of MEK pathway, the duration and the intensity of the signal is important, the most characteristic example being that of PC12 cells, where duration of ERK activity determines whether the cells proliferate or differentiate (31). Importantly, we have detected a rapid and sustained activation of ERK1/2 regulated by MEK for more than 3 hours after TRAIL treatment and subsequently sustained overexpression of c-fos for more that 90 minutes. This is in contrast to transient activation of MEK pathway and c-fos expression after typical growth factor stimulation, reported in many cell types. These results are consistent with previous studies where it was found that TRAIL mediates FADD dependent overexpression of the c-Fos transcription factor (32).

Transformation by Ras renders resistant colon cells sensitive to TRAIL

To examine if the ability of MEK signaling pathway mediates TRAIL effect in colon cells, we developed clones of colon cells overexpressing oncogenic ras forms, since Ras activates MEK pathway in many cell systems. Hence, we established stable Caco-2 cell lines constitutively expressing Ki-RasV12 (Caco-KV12) or Ha-RasV12 (Caco-HV12), and a control cell line stably transfected with the empty expression cassette (Caco-Neo). Caco-2 cells are an intermediate adenoma colon-derived cell line, which are often used in the study of enterocyte differentiation as upon reaching confluency the cells differentiate into a mature enterocyte phenotype. These cells bear no mutations in any of the three ras loci, and nor are they known to bear mutations in any of the genes that function as Ras effectors (e.g. B-Raf). Therefore, Caco-2 cells represent an ideal model system in which to introduce Ras mutations and examine their effects on MAP kinase signaling. Isolation and characterization of Ras overexpressing clones have been described elsewhere (Roberts et al., under revision ). Briefly, we had demonstrated that constitutively active RasV12 isoforms transform Caco-2 cells indicated by growth in soft agar and formation of tumors in SCID mice. Moreover, only Ha-RasV12 was capable of inducing an epithelial to mesenchymal transition, when compared to Ki-RasV12 and these findings were
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Ras overexpression converted the TRAIL-resistant Caco2 cells to cells responsive to TRAIL tumoricidal activity and especially in the case of Ha-ras overexpressing clones, in a more dramatic way. Tumor cells can become resistant to TRAIL by distinct mechanisms. We examined the reasons of induced sensitivity to cell death by Ras overexpression in these cell lines. We analyzed expression levels of TRAIL downstream components like FADD, caspases 3 and 8 and but no changes related to TRAIL sensitivity were detected. Expression levels of DR4 and DR5 were increased in a MEK dependent manner. Of special interest is the slower migrating form of DR5, which is predominantly expressed in the Ha-ras overexpressing clone, the most sensitive of all the cell lines tested to TRAIL induced apoptosis. In addition, the Ha-ras transformed clones presented a dramatic overexpression of the Fas receptor but this was not correlated to MEK activation. These findings argue that colon cells transformed by ras oncogene can be sensitive to TRAIL induced cell death in a MEK dependent manner. Overexpression of the Ha-ras oncogene has a better apoptosis sensitization potential in general, potentially since it strongly upregulates the Fas receptor as well. Recently, Ras sensitization of human embryonic kidney and foreskin fibroblast cells to tumoricidal activity of TRAIL has been reported (34), although not correlated to TRAIL receptor expression and MEK signalling pathways. We believe that the presence of oncogenic Ras acts upon the cells sensitivity to TRAIL through MEK signaling and through oncogenic transformation in cooperation with other oncogenes The latter could be one of the reasons that the Ha-ras transformed clones have higher sensitivity to TRAIL since oncogenic Ha-ras is known to have a greater transforming potential as compared to Ki-ras. In our model system, which is relevant to human colorectal cancer, a mechanistic explanation for the observed effect of ras oncogene is provided. Transformation with the c-myc oncogene (35) provides resistant cells with cell death sensitivity as well, so the presence of specific activated oncogenes can provide an explanation of sensitivity of tumor cells to TRAIL induced apoptosis in general.

Ras as a determinant factor for TRAIL specificity in killing tumors

Careful selection of therapeutic strategy based on molecular phenotyping of the patient’s tumor is evidently to become a major issue in the development of targeted anticancer therapeutics. The soluble recombinant TRAIL as well as monoclonal antibodies, which mimic its effect, are of interest for cancer therapy and are already in clinical trials (36, 37). Recent studies in the mouse suggest that TRAIL immunoselects tumors for TRAIL resistance (38). Data showing the impressive selective anti-tumor activity of soluble TRAIL in vitro have generated considerable excitement and have resulted in the development of TRAIL as a novel anti-cancer agent; only recently though a few key studies have addressed the natural role of TRAIL in immunity against cancer. Importantly, not all cancer cells are sensitive to the cytotoxic effects of TRAIL. Moreover, abnormalities of various components of death receptor pathways have been identified in human cancer including loss of Fas expression, deletion or loss of TRAIL receptor DR4, mutation of TRAIL receptor DR5, overexpression of TRAIL decoy TRID or overexpression of Fas decoy, as well as overexpression of, the activated caspase 8/10 inhibitor, FLIP. These changes can determine the resistance or sensitivity of a particular cancer cell type to TRAIL induced apoptosis (summarized in Ozoren and El-Deiry, 2003). In this study we have used an in vitro system based on human colorectal adenocarcinoma cells and we have shown that the presence of an activated form of ras oncogene can shift the balance of a resistant colon cell line towards sensitivity to TRAIL induced apoptosis by upregulating DR4 and DR5. In support of this hypothesis, cell lines originally bearing a mutant Ki-ras allele that was disrupted (40) had reduced TRAIL receptor levels (Fig. 7D). Although in our cell system the presence of activated ras can provide sensitivity to TRAIL, it is possible that in human tumors other genetic changes of TRAIL downstream components as those mentioned above can also contribute to sensitivity and this hypothesis will be tested in the future. We have also demonstrated that it is specific transformation by ras that renders colon cell sensitive, since MEK pathway is activated in this system and its inhibition prevents TRAIL induced death. It is likely that other oncogenes capable of activating the MEK pathway may
Ras sensitizes colon cells to apoptosis by TRAIL potentially provide these same properties to tumor cells. On the other hand, the PI-3K survival pathway does not play an important role on the sensitivity to TRAIL between the Ki- and Ha-ras clones. The findings of our study provide a mechanistic basis for a pharmacogenomic approach and could be further exploited therapeutically. More than half of all human colon cancers bear oncogenic mutations on ras genes (24). TRAIL and TRAIL agonists can potentially be used in order to destroy tumors that bare ras or other oncogenic mutations, which result in DR4 and DR5 receptor overexpression. Other approaches of inhibiting components of Ras pathways are also currently in process and have already provided agents with encouraging anticancer effects. Therefore, it is essential that careful therapeutic strategy selection should be made, as the combination of molecules inhibiting Ras pathway components with TRAIL agonists may not give the desired synergistic effect. In the recent years, efforts are made towards the generation of “smart” anticancer drugs that will target specific molecules depending on the molecular phenotyping of the patient’s tumor and many of these potential or current drugs influence signaling pathways. This raises the problem that an incorrect selection of multiple drugs could give conflicting results if the cross-talk of those pathways is not adequately taken into consideration.

ACKNOWLEDGEMENTS

This work was supported, in part by European Union Research Grants and the General Secretariat for Research and Technology of Greece to A.P., also by the grants KJB 5052407 (GA AS CR) to L.C., and K5020115 (AS CR) to L.A.. M.L.R. is the recipient of a EU Marie Curie Fellowship. We especially thank Era Taoufik (Hellenic Pasteur Institute, Athens, Greece) for critical reading of the manuscript.

REFERENCES

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FOOTNOTES

The abbreviations used are: TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; DR, Death Receptor; MAP, mitogen-activated protein; ERK, extracellular-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; FADD, Fas-associated death domain protein; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; FBS, fetal bovine serum; PI, propidium iodide; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEM, minimal essential medium; PARP, poly(ADP-ribose) polymerase; FACS, fluorescence-activated cell sorter; GAM, goat anti-mouse;

FIGURE LEGENDS

FIG. 1 Response of human colon adenoma-carcinoma cell lines to TRAIL treatment and steady state protein levels of apoptotic factors. A. The cells were incubated with 500ng/ml TRAIL and viability measured after 24hr. B. HT29 cells were incubated for 12h with or without 500ng/ml TRAIL, the nuclei were stained with Hoechst and Propidium Iodide and 500 cells from random fields were checked for apoptotic or necrotic nuclei. The arrows indicate apoptotic nuclei. AP: number of apoptotic nuclei; an insignificant number of necrotic nuclei was found and is not shown. At the same time, whole cell lysates were checked with WB for characteristic PARP cleavage. A representative western blot is shown. C. The relative mRNA levels of DR4 and DR5 in Caco-2, DLD-1 and HT29 cells were analyzed by RT-PCR and normalized to GAPDH; a representative image of 4 experiments is shown. D. The relative protein levels of FADD, Caspase-8 and Caspase-3, DR4 and DR5 were also analyzed by Western Blot and normalized to total ERK2 levels.

FIG. 2 TRAIL induces a rapid and sustained MEK dependent ERK1/2 phosphorylation and c-fos expression. A. HT29 and DLD-1 cells were treated with TRAIL and ERK1/2 (upper panel) and MEK activation (lower panel) was measured by Western Blot against the phosphorylated protein. Level of activity was determined after normalization to total ERK2 levels. B. c-fos mRNA levels were measured by RT-PCR using specific PCR primers from 30 to 90 min after TRAIL/PD98059 treatment, the values derived from multiple experiments, were all normalized to GAPDH and are represented graphically relative to 0 min time point; a representative image for the 30min induction is shown.

FIG. 3 Effect of MEK inhibition on TRAIL’s ability to induce apoptosis

HT29 cell line was incubated with 500ng/ml TRAIL and either pre-incubated for 16 h with 100µM MEK inhibitor PD98059 or not. A. Cell viability assay on the HT29 cell line showing the effect of the MEK inhibitor on TRAIL induced cell death in relation to the respective controls. B. WB analysis of PARP after pre-incubation with the MEK inhibitor prior to induction with 500ng/ml TRAIL for 12hr. C. HT29 cell line was incubated for 12 h with 500ng/ml TRAIL with or without preincubation with 100µM of the MEK inhibitor PD98059. The nuclei were stained with Hoechst and Propidium Iodide (data not shown) and 500 cells from random fields were checked for apoptotic or necrotic nuclei. The arrows indicate apoptotic nuclei. AP: apoptotic nuclei.

FIG. 4 Impact of MEK inhibition on protein and mRNA levels of the apoptosis-inducing TRAIL receptors, DR4 and DR5

A. RT-PCR was performed on RNA extracted from HT29 and DLD-1 cells that were either incubated 4h with 100µM of the MEK inhibitor, 500ng/ml of TRAIL or left untreated by using DR4 and DR5 specific primers. B. DR4 and DR5 protein levels were examined with or without 16h incubation with 100µM of the
Ras sensitizes colon cells to apoptosis by TRAIL

MEK inhibitor, reproducible changes were only observed on the DR protein levels by using specific antibodies. Levels of caspase 3 and 8 and FADD did not reveal significant changes (data not shown). All values were normalized to ERK2 protein and the graph, representing DR4 and DR5 expression levels change relative to the respective untreated cells, was generated from values obtained from 3 independent experiments.

FIG. 5 DR4 and DR5 levels on the cell surface - Effect of MEK inhibition
FACS analysis was performed on the HT29 cell line treated with 100µM PD98059 for 3h and 16h, the numbers on the first column show the levels of the expression of DR4, DR5, TRAIL-R3, TRAIL-R4 and Fas on the surface of the HT29 cells, using specific antibodies for each receptor, while the numbers on the second and third column show the percentage difference after 3h and 16h of PD98059 treatment respectively. The numbers of the first row represent the background signal when only the secondary antibody is used (GAM-PE).

FIG. 6 Ras oncogenes sensitize the Caco-2 cell line to TRAIL induced apoptosis
A. The cells were incubated with 1µg/ml TRAIL for up to 62h and viability measured at the time points indicated on the graph that shows percentage of viable cells. B. The cells were incubated for 12h with or without 1µg/ml TRAIL and whole cell lysates were checked by WB for characteristic PARP cleavage. C. Western Blot analysis of steady state levels of p-Akt in the ras transformed clones and after 2.5h treatment with 200nM of the PI3-K inhibitor Wortmannin. All values are relative to Neo p-Akt steady state levels. D. Cell viability assay on the ras transformed Caco-2 cells after 48h induction with TRAIL with or without Wortmannin. The bars represent percentage of viability relative to respective controls.

FIG. 7 Impact of ras oncogenes overexpression on DR4 and DR5 mRNA and protein expression levels
A. RT-PCR performed on RNA extracted from the clones. The values were normalized to GAPDH and showed that mRNA levels of DR4 and DR5 were higher on both the Ha-ras and Ki-ras clones; a representative image of 4 experiments is shown. B. The relative levels of DR4 and DR5 were checked by Western Blot. C. To determine the actual expression and localization of the receptors on the surface of the membrane, FACS analysis was performed on the clones using specific antibodies showing a clear upregulation of both receptors in both clones. D. RT-PCR analysis of DR4 and DR5 steady state mRNA levels of DKO-4 cells and the parental DLD-1 cells transfected with the control cassette. A representative image is shown.

FIG. 8 Consequences of MEK inhibition on protein and mRNA levels of the apoptosis-inducing TRAIL receptors in the ras transformed clones
A. Steady state phosphorylated levels of ERK1/2 proteins and the effect of the PD98059 on these levels was determined Western Blot analysis using phosphor-specific antibodies against ERK-1/2.. B. DR4 and DR5 mRNA levels were analyzed by RT-PCR and was performed on RNA extracted from cells that were either incubated 5h with 100µM of the MEK inhibitor or not, and the values were normalized to GAPDH and shown below each lane; a selected representative image from 4 repeats is shown. C. DR4 and DR5 protein levels were examined by western blot analysis with or without 16h incubation with 100µM of the MEK inhibitor. D. A viability assay was performed on the H2 clone with or without 16h pretreatment with PD98059. The graph represents relative to respective control decrease in viability starting from the moment TRAIL was added to the growth medium.
Figure 1

A

![Graph showing % viability of different cell lines after 24h treatment with Control and Trail 500ng/ml.](image)

B

![Images of cell viability and PARP expression](image)

Control
AP: 3

12h
AP: 129

AAC1, RGC2, CACO-2, DLD-1, HT-29

% viability

PARP

Control 12 h

112kD 85kD
Figure 1

Caco2  DLD-1  HT29

**C**

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<th>DLD-1</th>
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**D**

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Figure 2

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|------------|----------|----------|----------|----------|      |----------|----------|----------|----------|
|            | p-MEK    |          |          |          |      |          |          |          |          |
| HT29       |          | Control  | 2 min    | 5 min    | 10 min|          | Control  | 2 min    | 5 min    | 10 min   |
|            |          | 1        | 1.5      | 2        | 1.6   |          | 1        | 1.4      | 1.9      | 1.6      |

44kD 42kD 45kD
Figure 2

B

HT29
30min
TRAIL  -  +  +
PD      -  +  -
c-FOS
1  1.2  2.1

GAPDH
1  1  1.1

HT29 c-FOS mRNA

TRAIL  ←  TRAIL + PD

Fold

0min  30min  60min  90min
Figure 3

A

![Graph showing viability of HT29 cells with TRAIL and PD/TRAIL](image)

B

![Western blot of PARP with TRAIL and PD](image)
Figure 3

C

Control

PD98059

TRAIL

TRAIL/PD98059
Figure 4

A

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B

HT29

Normalised to ERK2

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Fold

0 0.2 0.4 0.6 0.8 1 1.2

0 0.2 0.4 0.6 0.8 1 1.2

0 0.2 0.4 0.6 0.8 1 1.2

0 0.2 0.4 0.6 0.8 1 1.2
Figure 5

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Figure 6

A

![Graph showing viability of Neo, K15, H2, and H13 cells over time.](https://via.placeholder.com/150)

B

![Image showing Western blots for Neo and H2 with and without TRAIL treatment.](https://via.placeholder.com/150)

C

![Image showing Western blots for p-AKT and ERK2 with Wortmannin treatment.](https://via.placeholder.com/150)
Figure 6

D
Figure 7

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27kD  50kD
55kD  48kD
33kD  43kD
42kD
Figure 7

C

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D

DLD-1  DKO-4

DR4

1  0.7

DR5

1  0.7

GAPDH

1  1
Figure 8

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Figure 8

C

Normalised to ERK2

D

% viability

H2

Trail
Trail - PD

Control 24h 48h
Transformation by oncogenic Ras sensitizes human colon cells to TRAIL induced apoptosis by upregulating DR4 and DR5 receptors through a MEK-dependent pathway

Konstantinos G. Drosopoulos, Michael L. Roberts, Lukas Cermak, Takehiko Sasazuki, Senji Shirasawa, Ladislav Andera and Alexander Pintzas

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