Transformation by Oncogenic RAS Sensitizes Human Colon Cells to TRAIL-induced Apoptosis by Up-regulating Death Receptor 4 and Death Receptor 5 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, 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 specific 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 Ki- and mainly by Ha-ras oncogenes sensitizes these cells to TRAIL-induced apoptosis by causing specific MEK-dependent up-regulation of DR4 and DR5. These observations taken together reveal that RAS-MEK-ERK1/2 signaling pathway can sensitize cells to TRAIL-induced apoptosis by up-regulating 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, and 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 1 (Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand) is a cytokine that can selectively induce apoptosis in tumor cells although leaving normal cells largely unaffected. The effects of TRAIL are mediated through the tumor necrosis factor receptor superfamily members, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). TRAIL can also bind to the decoy receptors, the role of which remains to be identified (3, 4). There are four distinct TRAIL receptors, all belonging to the tumor necrosis factor receptor superfamily: TRAIL-R1 (DR4) (5), TRAIL-R2 (DR5) (6), TRAIL-R3 (TRID/DcR1/lymphotoxin inhibitor of TRAIL (LIT)), and TRAIL-R4 (DcR2) (7). The latter two are incapable of transducing the signal because they either do not contain a death domain or contain a truncated death 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 signaling complex. At this level, procaspases undergo autocatalytic processing to be activated, leading to the activation of the effector caspases (11), mitochondrial dysfunction, and ultimately apoptosis.

Even though TRAIL receptor activation is mainly linked to the induction of apoptosis, evidence implicates TRAIL signaling in the activation of c-Jun NH₂-terminal kinase (12), NFκB (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 up-regulation 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 up-regulation. Similarly TRAIL-R2 has been shown to be up-regulated through NFκB (17). These results taken together allow us to assume that molecules not directly implicated in the TRAIL pathway play an important role in this process.

1The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; DR, death receptor; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAP, mitogen-activated protein; FADD, Fas-associated death domain protein; GADD, glyceraldehyde-3-phosphate dehydrogenase; FARP, poly-(ADP-ribose) polymerase; FACS, fluorescence-activated cell sorter; RT, reverse transcription; WB, Western blot; PI3K, phosphatidylinositol 3-kinase; p-, phosphorylated; DcR, decoy receptor; TRID, TRAIL receptor without an intracellular domain.
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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 (22). Studies in mice that genetically modified to express ras oncogenes have very clearly shown that activated Ras promotes the initiation, progression, and maintenance of several types of tumors (23, 24). 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. The activated ERK1/2 translocate to the nucleus and regulate pathways like MEK, PI3K, and Rho GTPases. The MEK pathway has been shown to be active in human colon adenocarcinoma cells (25) as well as in human colorectal tumors (26). The activated ERK1/2 translocate to the nucleus and regulate the expression of target genes like c-FOS and/or FRA-1 (27, 28).

Sensitization to TRAIL-induced apoptosis very often is mediated by up-regulation of TRAIL receptors DR4 and DR5 (29). Interestingly, expression of DR4 and DR5 has been found elevated in human colon carcinomas as compared with normal colonic mucosa (30); we aimed to pinpoint oncogenic pathways that make TRAIL more efficient against cells representing the broad range of human cancer cells and tumor regression in mice without significant systemic toxicity (4). In addition, TRAIL can complement current strategies because it can render death sensitivity to tumors by its synergistic action with chemotherapeutic drugs or UV treatment (20, 21).

FACS Analysis—For immunostaining, 5 × 10^5 cells were preincubated with blocking buffer (phosphate-buffered saline containing 0.2% gelatin, 0.1% sodium azide, and 20% fetal bovine serum) and then incubated with the staining buffer (phosphate-buffered saline containing 0.2% gelatin and 0.1% sodium azide) with monoclonal antibody (50 μg/ml) on ice for 30 min. After washing, cells were incubated in the staining buffer containing phycoerythrin-conjugated goat anti-mouse (no. 1070-09, Southern Biotechnology Associates). Cells were then washed, resuspended in the staining buffer, and analyzed by flow cytometry. The following antibodies were used for FACS analysis: DR4 (Santa Cruz Biotechnology Inc., sc-6131); DR5 (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-7384); caspase-3 (Santa Cruz Biotechnology Inc., sc-7384); GAPDH (Santa Cruz Biotechnology Inc., sc-7384). All PCR products were normalized to GAPDH expression. RT-PCR—RNA isolation was performed using the TRIzol reagent (Invitrogen). cDNA was prepared using an oligo(dT) primer and Molostrak reverse transcriptase (Promega) following standard protocols. Primers used in these experiments were as follows: TRAIL-R1, 5'-CAGAACGTCCTGGACCTGTAAC-3' and 5'-AGTGTCAATTGCTGATTTG-3'; TRAIL-R2, 5'-GGGAAAGAATTCCTGAGAG-3' and 5'-ACATTGTCCTGACCCCAAGCT-3'; GADD45, 5'-ACACAGTGTCACATGACAC-3' and 5'-TCCACCCCTTGTCGTTA-3'; c-FOS, 5'-GAATAAGATGGCTGCAGCAAATGC-3' and 5'-ATGGCTGTCGAACTTGAAGAC-3'; GAPDH, 5'-ACAGGAACTCTGTAAGCGTGACAG-3' and 5'-GAAGTCGTTGAACTGTCAG-3'.

Cytotoxity and Apoptosis Assays—For cell viability assays, cells were plated on 6-well plates, fixed with absolute methanol, stained with 0.5% crystal violet for 10 min, and washed three times with phosphate-buffered saline; the remaining crystal violet was extracted using 30% acetic acid and absorbance was measured at 595 nm. 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 DNA binding assay (Sigma). After reverse transcription the cDNA product was amplified by PCR with 3 units of TaqDNA polymerase (Promega) and 2.5 μM Mg²⁺ using standard protocols; the annealing temperature was 56 °C for all of the 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 a UV transilluminator. Intensity values were measured using ImageQuant software (Amersham Biosciences). All PCR products were normalized to GADD45 expression.

Materials and Methods

Cell Cultures—The Caco-2, DLD-1, and HT29 cells were obtained from ATCC. The human colon cell lines Caco-2 and HT-29 and the RAS-overexpressing clones derived from Caco-2 were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), antibiotics, and nonessential amino acids (Invitrogen). The human colon adenocarcinoma cell line DLD-1 and the derived clone that has the oncogenic Ki-RAS allele disrupted (DKO-4) (31) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics. The human colon cell lines AAC1 and RGC2, obtained from Professor C. Paraskeva (United Kingdom), were cultured in the same manner as the HT-29 cells with the addition of insulin, hydrocortisone, and 20% fetal bovine serum (Invitrogen). RGC2 and AAC1 are clonogenic, nonmalignant human colonic adenoma-derived cell lines (32). The MEK inhibitors PD98059 and U0126 were purchased from Alexis Biochemicals. The PI3K inhibitor wortmannin was purchased from Sigma. The Caco-2 clones constitutively expressing active RAS proteins will be described in a forthcoming article. 2 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 (Invitrogen). The colonies expressing between 2- and 3-fold expression in were Bio-Rad 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.

Cytokines and Culture Conditions—Cells were cultured in 25 cm² tissue culture flasks (Falcon), in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FCS, 100 units/ml of penicillin and streptomycin, and 10 μg/ml of gentamycin. The RPMI medium was obtained from Invitrogen. The Caco-2, DLD-1, and HT29 cell lines were obtained from ATCC. Cells were maintained in culture with medium changes every 48 h. The medium was replaced with medium containing 0.5 μg/ml of TRAIL (Bedford Discovery Research). The Caco-2, DLD-1, and HT29 cells were obtained from ATCC. Cells were maintained in culture with medium changes every 48 h. The medium was replaced with medium containing 0.5 μg/ml of TRAIL (Bedford Discovery Research).
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 500 ng/ml TRAIL, and viability was measured after 24 h. B, HT29 cells were incubated for 12 h with or without 500 ng/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 four experiments is shown. D, the relative protein levels of FADD, caspase-8, caspase-3, DR4, and DR5 were also analyzed by Western blot and normalized to total ERK2 levels.

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ERK2 protein expression and/or Ponceau staining was used for protein loading control; ERK2 was also used for normalization. All of the experiments were repeated at least three times, and S.D. function was used for error bar generation. All controls in every experiment were treated with the same amount of buffer/diluent as 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, whereas non-tumor cells have been reported to be resistant to TRAIL-induced death in many systems (4). 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 periods of TRAIL treatment. The three colon adenoma cell lines examined in the study, AAC1 (32), RGC2, and Caco-2, showed no difference in viability after a 24-h treatment with 500 ng/ml TRAIL (Fig. 1A). Even after 72 h 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 in Fig. 1B. Similar results were obtained with the DLD-1 cell line (data not shown). The expression levels of the functional TRAIL receptors and 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 with Caco-2 cells that were correlated with 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 the 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 signaling pathways like c-Jun NH2-terminal kinase (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 500 ng/ml TRAIL. ERK phosphorylation followed MEK activation that peaked after ~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 90 min after treatment with TRAIL, although this increase was abolished by co-treatment with the MEK inhibitor PD98059 (Fig. 2B). Remarkably, c-FOS induction by TRAIL was sustained for 90 min 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 25 min of incubation with TRAIL, an 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, whereas MEK Inhibition Reduces the Ability of TRAIL to Induce Apoptosis by Down-regulating Their Expression—Incubation with TRAIL has been reported to activate a positive feedback loop causing the up-regulation of TRAIL-R2 (DR5) (17), and this effect contributes to 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. Pretreatment of the HT29 cell line for 16 h with 100 μM MEK inhibitor PD98059 prevented TRAIL from significantly reducing cell viability (Fig. 3A) and from inducing apoptosis. Specifically, HT29 cells treated for 24 h with TRAIL showed significant reduction in viability relative to the control. Pretreatment for 16 h with the MEK inhibitor of cells to be treated with TRAIL resulted in an 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% (mean ± S.D.) of the nuclei counted in the cells treated with TRAIL had apoptotic characteristics, whereas 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).

Additional evidence for the regulatory role of the 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. RT-PCR was performed on RNA extracted from HT29 and DLD-1 cells that were incubated 4 h with a 100 μM concentration of the MEK inhibitor PD98059 (PD) or 500 ng/ml TRAIL or were left untreated by using DR4- and DR5-specific primers. B, DR4 and DR5 protein levels were examined with or without a 16-h incubation with 100 μM MEK inhibitor; reproducible changes were observed only on the DR protein levels by using specific antibodies. Levels of caspase-3 and-8 and of FADD did not reveal significant changes (data not shown). All of the values were normalized to ERK2 protein, and the graph, representing DR4 and DR5 expression level changes relative to the respective untreated cells, was generated from values obtained from three 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 100 μM PD98059 (PD) for 3 and 16 h. The numbers in the first column (control, CTRL) 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, and the numbers in the second and third columns show the percentage difference after 3 and 16 h of PD98059 treatment, respectively. The numbers of the first row represent the background signal when only the secondary antibody is used (GAM-PE, goat anti-mouse-phycoerythrin). O/N, overnight.
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 of the TRAIL receptors increase after treatment with TRAIL (Fig. 4A). To determine whether the actual expression levels of the DR4 and DR5 receptors were altered on the cell surface, we performed FACS analysis on the HT29 cells and confirmed the observations made by Western blot and RT-PCR. There was a slight decrease in the cell surface levels of DR4 and DR5 after a 1-h incubation with MEK inhibitor PD98059, whereas a 16-h incubation showed a ~42 ± 4.2% and a 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 receptor levels in the cells under investigation (Fig. 5).

Human Colon Cell Transformation by Oncogenic Ki- and Ha-RAS Up-regulates 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 nontransformed human cells to TRAIL-induced apoptosis, we examined whether 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, the Ha-RASV12, and the empty vector (Neo) as a control,2 and clones with low-to-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 (Fig. 6A, H2 and H13) showed responsiveness to TRAIL in reducing cell viability by ~35% after 24 h and by

FIG. 6. RAS oncogenes sensitize the Caco-2 cell line to TRAIL-induced apoptosis. A, the cells were incubated with 1 μg/ml TRAIL for up to 62 h, and viability was measured at the time points indicated on the graph, which shows the percentage of viable cells. Neo, empty vector as a control; K15, Ki-RAS; H2 and H13, Ha-RAS-transformed cells. B, the cells were incubated for 12 h with or without 1 μg/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 2.5 h of treatment with a 200 nm concentration of the PI3K 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 48-h induction with TRAIL with or without wortmannin. The bars represent percentage of viability relative to respective controls.
73% after 62 h of treatment, whereas the Ki-RAS (Fig. 6A, K15) clones were more resistant; beginning to show significantly reduced viability after 62 h of treatment (Fig. 6A); these clones gave representative results of the various neo-transformed and 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 PARP cleavage (Fig. 6B). Moreover, we examined the steady state levels of phosphorylated Akt at Ser-473, a downstream effector of PI3K survival signaling, to see whether 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 PI3K signaling (Fig. 6C) as reported previously in other systems (33). Using a PI3K 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 PI3K inhibition allowing us to assume that it is unlikely that PI3K signaling plays an important role in the differential responsiveness of the clones to TRAIL.

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 with the control (neo)-transfected cells (Fig. 7A). Western blot analysis of the expression levels of DR4 and DR5 showed a 1.7-fold up-regulation of these receptors on average, whereas 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 50 kDa (Fig. 7B). Overexpression of DR4 and DR5 in the Caco-2 RAS-transformed cells was confirmed on the cell surface by FACS analysis (Fig. 7C). In addition to DR4 and DR5 up-regulation, we noted that in the case of the Ha-RAS clones there is a strong up-regulation and cell surface localization of the Fas receptor as analyzed by FACS (Fig. 7C), whereas in both cases the decoy receptor levels were not altered as compared with the control Caco-2 neo clones. To further confirm the effect of oncogenic RAS on DR4 clones.
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 (31). 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 on 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 the MEK inhibitor PD98059 prior to treatment with TRAIL (Fig. 8D). We note that these results were reproducible when we used the UO126 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 than that observed in traditional therapies. As such, the most attractive therapeutic agent would target regulated cell properties such as survival, cell cycle, signal transduction, and metastasis. Studies with this approach are currently in progress to predict which targets and therapeutic candidates will be most promising in the treatment of colorectal cancer (34).

Constitutively activated Ras increases the tumorigenic potential of cells because 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 the incidences of colorectal cancer. 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, FAS ligand, 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.

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

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**Fig. 7. Impact of RAS oncogene 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 four experiments is shown. Neo, empty vector as a control; K15, Ki-RAS; H2 and H13, Ha-RAS-transformed cells. B, the relative levels of DR4 and DR5 were checked by Western blot. Casp, caspase. 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 up-regulation of both receptors in both clones. CTRL, control. 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.
DR5 were down-regulated, 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 nonresponsive to TRAIL-induced apoptosis ERK1/2 activation by overexpression of activated RASV12 isoforms consequently up-regulated expression of DR4 and DR5 and sensitized the cells to death, whereas disruption of oncogenic Ki-RAS reduced both the phosphorylation levels of ERK proteins and death receptor 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 up-regulation of the death receptor 5 gene is dependent on NFκB (17) and AP-1 (18). Therefore, TRAIL receptor expression as well as the sensitivity of cells to TRAIL is regulated by multiple factors besides MEK, 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 with a B-RAF mutation in HT-29 cells, as well as a different APC mutation than HT-29 cells, which results in differential signaling to the nucleus (35). These differences, 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 depend on the balance of signaling pathways; in the case of the MEK pathway the duration and the intensity of the signal is important, the most characteristic example being that of PC12 cells, in which the duration of ERK activity determines whether the cells proliferate or differentiate (36). Importantly, we have detected a rapid and sustained activation of ERK1/2 regulated by MEK for more than 3 h after TRAIL treatment and a subsequently sustained overexpression of c-FOS for more than 90 min. This is in contrast to the 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 in which it was found that TRAIL mediates FADD-dependent overexpression of the c-FOS transcription factor (37).
Transformation by Ras Renders Resistant Colon Cells Sensitive to TRAIL—To examine whether the ability of the MEK signaling pathway mediates the TRAIL effect in colon cells, we developed clones of colon cells overexpressing oncogenic ras forms because Ras activates the 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 from an intermediate adenoma colon-derived cell line that is often used in the study of enterocyte differentiation because upon reaching confluency the cells differentiate into a mature enterocyte phenotype. These cells bear no mutations in any of the three RAS loci, 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.

The isolation and characterization of RAS-overexpressing clones will be described in a forthcoming article. Briefly, we have demonstrated that constitutively active RASV12 isoforms transform Caco-2 cells indicated by growth in soft agar and the formation of tumors in severe combined immunodeficient mice. Moreover, only Ha-RASV12 was capable of inducing an epithelial to mesenchymal transition when compared with Ki-RASV12, and these findings were further supported by gene expression profile analysis.

RAS overexpression converted the TRAIL-resistant Caco-2 cells to cells responsive to TRAIL tumoricidal activity and, especially in the case of Ha-RASV12-overexpressing clones, in a more dramatic way. Tumor cells can become resistant to TRAIL by distinct mechanisms. We examined the reasons for induced sensitivity to cell death by RAS overexpression in these cell lines. We analyzed expression levels of TRAIL downstream components like FADD and caspases 3 and 8, 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 with 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 because it strongly up-regulates the FAS receptor as well. Recently, RAS sensitization of human embryonic kidney and foreskin fibroblast cells to tumoricidal activity of TRAIL has been reported (38), although it has not been correlated with TRAIL receptor expression and MEK signaling pathways. We believe that the presence of oncogenic RAS acts upon the sensitivity of the cells 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 because oncogenic Ha-RAS is known to have a greater transforming potential as compared with 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 (39) pro-
RAS Sensitizes Colon Cells to Apoptosis by TRAIL

RAS as a Determining 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 cancer therapeutics. The soluble recombinant TRAIL, as well as monoclonal antibodies that mimic its effect, are of interest for cancer therapy and are already in clinical trials (40, 41). Recent studies in the mouse suggest that TRAIL immunoextracts tumors for TRAIL resistance (42). 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 anticancer agent; only recently, moreover, 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, and overexpression of Fas decoy receptor, 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 (43). 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 toward sensitivity to TRAIL-induced apoptosis by up-regulating DR4 and DR5. In support of this hypothesis, cell lines originally bearing a mutant Ki-RAS allele that was disrupted (31) 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, such as those mentioned above, can also contribute to sensitivity, and this hypothesis has been validated in the future. We have also demonstrated that it is specific transformation by RAS that renders colon cells sensitive because the 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 potentially provide these same properties to tumor cells. On the other hand, the P13K survival pathway does not play an important role in the sensitivity to TRAIL on the part of 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 (22). TRAIL and TRAIL agonists can potentially be used to destroy tumors that bear RAS or other oncogenic mutations that cause DR4 and DR5 receptor overexpression. Other approaches to inhibiting the components of TRAIL pathways are also currently in process and have already provided agents that have encouraging anticancer effects. Therefore, it is essential that careful therapeutic strategy selection should be made because the combination of molecules inhibiting RAS pathway components with TRAIL agonists may not give the desired synergistic effect. In recent years, efforts have been made toward the generation of “smart” anticancer drugs that will target specific molecules depending on the molecular phenotype 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.

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