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Olivier Meurette, Amélie Rébillard, Laurence Huc, Gwenaëlle Le Moigne, Delphine Merino, Olivier Micheau, Dominique Lagadic-Gossmann, Marie-Thérèse Dimanche-Boitrel

TRAIL Induces Receptor-Interacting Protein 1–Dependent and Caspase-Dependent Necrosis-Like Cell Death under Acidic Extracellular Conditions

Olivier Meurette,[1] Amélie Rebillard,[1] Laurence Huc,[1] Gwenaëlle Le Moigne,[1] Delphine Merino,[2] Olivier Micheau,[2] Dominique Lagadic-Gossmann,[1] and Marie-Thérèse Dimanche-Boitrel[1]

[1]Institut National de la Santé et de la Recherche Médicale UMR620, Université Rennes 1, Rennes, France and [2]Institut National de la Santé et de la Recherche Médicale U517, Université Bourgogne, Dijon, France

Abstract

Tumor necrosis factor-α–related apoptosis-inducing ligand (TRAIL) is a potential anticancer agent that induces apoptosis in cancer cells but not in most normal cells. How tumor physiology, particularly acidic extracellular pH (pH_e), would modify sensitivity of cancer cells to TRAIL-induced cell death is not known. We have previously shown that cancer cells, resistant to TRAIL-induced apoptosis at physiologic pH_e (7.4), could be sensitized to TRAIL at acidic pH_e (6.5). However, at this acidic pH_e, cell death was necrotic. We show here that, in spite of a necrosis-like cell death morphology, caspases are activated and are necessary for TRAIL-induced cell death at acidic pH_e in HT29 human colon cancer cells. Furthermore, we observed that, whereas receptor-interacting protein (RIP) was cleaved following TRAIL treatment at physiologic pH_e (7.4), it was not cleaved following TRAIL treatment at acidic pH_e (6.5). Moreover, RIP degradation by geldanamycin or decrease expression of RIP by small RNA interference transfection inhibited TRAIL-induced necrosis at acidic pH_e, showing that RIP was necessary for this necrotic cell death pathway. We also show that RIP kinase activity was essential for this cell death pathway. Altogether, we show that, under acidic pH_e conditions, TRAIL induces a necrosis-like cell death pathway that depends both on caspases and RIP kinase activity. Thus, our data suggest for the first time that RIP-dependent necrosis might be a major death pathway in TRAIL-based therapy in solid tumors with acidic pH_e. [Cancer Res 2007;67(1):218–26]

Introduction

Cell death mainly occurs through two forms of cell death: apoptosis and necrosis. Apoptosis, also called "programmed cell death" (PCD), is characterized by the activation of caspases (cysteine proteases). Dying by apoptosis is an active process requiring energy in the form of ATP. At the opposite, necrosis is a passive process resulting from a bioenergetic catastrophe characterized by an important ATP depletion incompatible with cell survival. Accidental necrosis is characterized by cellular swelling, cytoplasm vacuolation, and plasma membrane breakdown. However, since several years, alternative, nonapoptotic forms of PCD have been described and classified as programmed necrosis or autophagy. Most of the time, necrosis-like PCD involves specialized caspase-independent signaling pathways, but in some cases, caspase-dependent signaling pathways have also been observed (1). Necrosis can be regarded as a controlled mechanism, which is involved in physiologic processes, such as embryogenesis, normal tissue renewal, and immune response (2). Necrosis has also been implicated in cancer therapy (3), although more investigations are still needed to establish the role of such a cell death (4).

Tumor necrosis factor (TNF)-α–related apoptosis-inducing ligand (TRAIL) is a potential anticancer agent that induces apoptosis in cancer cells but not in most normal cells (5). Depending on the cell type, death receptor triggering can induce apoptosis or necrosis (6, 7) and, depending on the stimulation of both apoptosis and necrosis, can occur in the same cell (8). Many reports have shown that death receptor ligation induces programmed necrosis under specific experimental conditions, such as intracellular ATP depletion (1, 9) and caspase inhibition (10, 11), or in caspase-8 deficient cells (12). More recently, a murine recombinant TRAIL molecule has been reported to naturally induce a caspase-independent necrosis in TRAMP-C2 murine prostate adenocarcinoma cells (13).

The necrotic program induced by death receptors is dependent on the receptor-interacting protein RIP1 (10). RIP1 protein contains three domains, including an NH_2-terminal serine/threonine kinase domain, an intermediate domain, and a COOH-terminal death domain (14). RIP1 has been involved both in nuclear factor-κB (NF-κB) activation (15, 16) and in cell death induction (10, 17, 18). Whereas the death domain and the intermediate domain of RIP1 are involved in NF-κB activation, the kinase domain of RIP1 is dispensable (see ref. 19 for review). However, this kinase domain is necessary for FasL-, TRAIL-, and TNF-induced caspase-independent necrosis (10).

We have recently reported that, under acidic extracellular conditions, TRAIL induces a caspase-dependent necrosis-like cell death (20). An acidic extracellular environment is often a characteristic of solid tumors (21), and evidence indicates that it might modulate the tumor response to chemotherapy *in vivo* (22). Furthermore, the role of pH in cell death is now well recognized (23). A recent work has shown that sensitization of human prostate or colon cancer cells to TRAIL-induced apoptosis under acidic extracellular conditions was dependent on mitochondrial apoptotic pathway (24). We show here that, under acidic extracellular pH (pH_e 6.5) conditions, HT29 human colon cancer cells are sensitized to TRAIL through a necrosis-like cell death, which is dependent on caspase activation. In contrast, under physiologic pH_e (7.4) conditions, TRAIL induces a typical apoptotic cell death. Moreover, degradation of RIP1 by geldanamycin pretreatment, small RNA interference inhibition of RIP expression, and transfection of

Requests for reprints: Marie-Thérèse Dimanche-Boitrel, Institut National de la Santé et de la Recherche Médicale UMR620, Faculté de Pharmacie, 2 Av du Pr Léon Bernard, 35043 Rennes, France. Phone: 33-2-23-23-48-37; Fax: 33-2-23-23-47-94; E-mail: marie-therese.boitrel@rennes.inserm.fr.

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kinase-dead RIP-expressing vectors inhibited TRAIL-induced necrosis under acidic extracellular conditions. Altogether, these data therefore show that, under acidic extracellular conditions, TRAIL induces a necrosis-like cell death that depends on both caspases and RIP1 kinase activity. This new TRAIL-induced necrosis-like PCD pathway may account for TRAIL-induced cell death in solid tumors with acidic pH_e.

Materials and Methods

Chemicals and antibodies. The recombinant human soluble Flag-tagged TRAIL was from Alexis Biochemicals (Coger, Paris, France).[3] Propidium iodide, anti-Flag M2, and membrane-permeable calpain inhibitor E64d were from Sigma-Aldrich (Saint-Quentin Fallavier, France). The dihydroethidium (DHE) and Hoechst 33342 were from Molecular Probes (Invitrogen, Abingdon, United Kingdom). TransFectin lipid and Bradford reagents were from Bio-Rad (Marnes-la-Coquette, France). Mouse anti-RIP was from PharMingen (BD Biosciences, Le Pont de Claix, France). The rabbit polyclonal anti-poly(ADP-ribose) polymerase (PARP) was from Roche (Roche Diagnostics France S.A., Meylan, France). The rabbit anti-phosphorylated IκB-α and the rabbit anti-IκB-α were from Cell Signaling (Ozyme, Saint Quentin en Yvelines, France). The mouse monoclonal anti-heat shock protein constitutive 70 (HSC70) and the rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were from Santa Cruz (Tebu, Le Perray en Yvelines, France). The rat anti-heat shock protein 90 (HSP90) was from Stressgen (Tebu). The anti-Flag M2 was used to cross-link the ligand TRAIL, leading to the formation of oligomers that are more effective to induce cell death. Briefly, 50 ng/mL of the soluble Flag-tagged TRAIL were incubated with 2 μg/mL anti-Flag M2 for 5 min at room temperature before cell treatment. The caspase-8 inhibitor (z-IETD-fmk), the caspase inhibitor negative control (z-FA-fmk, an inhibitor of cathepsin D), geldanamycin, CA-074-Me (cathepsin B inhibitor IV), calpain inhibitor III, and IκB-α phosphorylation inhibitor Bay 117085 were from Calbiochem (France Biochem, Meudon, France).

Cell culture conditions and cytotoxic assay. The HT29 human colon carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD) and cultured in Eagle's MEM (Eurobio, Les Ulis, France) supplemented with 10% (v/v) FCS (Life Technologies, Cergy Pontoise, France) and glutamine (2 mmol/L) under a 5% CO_2 atmosphere.

To modify the value of pH_e, a culture medium without sodium hydrogenocarbonate (Eurobio) was used. To set the pH_e of this medium to either 7.4 or 6.5, we next supplemented it with 23 or 3 mmol/L sodium hydrogenocarbonate, respectively, in an atmosphere of 5% CO_2.

Microscopic detection of apoptosis or necrosis was carried out in both floating and adherent cells recovered after TRAIL treatment using nuclear chromatin staining with 1 μg/mL Hoechst 33342 and 1 μg/mL propidium iodide for 15 min at 37°C. Apoptotic cells (i.e., with condensed blue chromatin or fragmented blue nuclei) or necrotic cells (i.e., with red nuclei) were counted in comparison with total population (n = 300 cells).

Cell transfection. HT29 cells (400,000) seeded in six-well plate were transfected with either nonspecific (nontargeting siRNA1, Dharmacon, Ozyme, Saint Quentin en Yvelines, France) or RIP siRNA (siGENOME siRNA gene ID 8737, Dharmacon) by using TransFectin according to the manufacturer's instructions. Briefly, 100 nmol/L siRNA was applied in a final volume of 1 mL Opti-MEM (Life Technologies). For Western blot analysis, cells were harvested 48 h after transfection. For cell death experiment, following 16-h transfection period, cells were treated with TRAIL at acidic pH_e for 24 h.

Plasmid transfections were done using Amaxa nucleofector (Amaxa GmbH, Cologne, Germany) according to the manufacturer's instructions. Briefly, trypsinized cells were resuspended in provided supplemented buffer and transfected with 3 μg of green fluorescent protein (GFP) plasmids alone or in combination with 3 μg RIP-expressing pMSCV plasmids. RIP wild-type (WT), RIP K45R, and RIP D138N plasmids were generated by PCR amplification and checked by sequencing. Twenty-four hours after transfection, cells were treated with TRAIL at acidic pH_e and analyzed by flow cytometry 24 h later to determine the percentage of propidium iodide–stained cell among the GFP-positive cells.

Cell morphology. Cells were fixed by dropwise addition of glutaraldehyde and analyzed according to standard conditions. After fixation, the specimens were rinsed several times with PBS followed by postfixation with 1% osmium tetroxide in phosphate buffer for 1 h. After a further rinsing again with PBS for 15 min, the tissue specimens were dehydrated through a series of graded ethyl alcohols from 70% to 100%. Cells were then embedded in DMP30 Eponate for 24 h at 60°C.

Measurement of caspase activities. Cell lysates (50 μg) obtained in radioimmunoprecipitation assay (RIPA) buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1% sodium dodecylsulfate, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L paramethylsulfonide, 1 mmol/L benzamidine] were incubated for 30 min at 37°C in a caspase assay buffer [100 mmol/L HEPES (pH 7.0), 10% glycerol, 1 mmol/L EDTA, 0.1% CHAPS, 1 mmol/L DTT] containing 20 μmol/L of either IETD-AFC or DEVD-AMC (Calbiochem). Caspase activities were measured by monitoring fluorescence continuously in a dual luminescence fluorimeter (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, CA) using specific excitation and emission wavelength for each peptide. Enzyme activities were determined as initial velocities and expressed as relative intensity/minute/milligram protein compared with control.

NF-κB activity measurement. NF-κB activation was measured with the TransAM NF-κB p65 kit (Active Motif, Rixensart, Belgium). The kit is an ELISA assay based on measurement of p65 binding activity to specific consensus DNA sequence. Briefly, nuclear extracts were purified as follows: cells were washed with PBS supplemented with phosphatase inhibitors and then resuspended in hypotonic buffer (20 mmol/L HEPES, 5 mmol/L NaF, 10 μmol/L Na_2MoO_4, 0.1 mmol/L EDTA). Cells were then kept on ice for 15 min before addition of 10% NP40 (0.5% final). After a 30-s centrifugation, the nuclear pellet was resuspended in complete lysis buffer and rocked for 30 min on ice. After a 10-min centrifugation at 14,000 × g, protein concentration was determined on the supernatant by Bradford assay. Nuclear extract (10 μg) was added per ELISA well, incubated with anti-p65 primary antibody for 2 h, washed, and incubated with the secondary peroxidase-conjugated antibody for 1 h. After three washes, the developing solution was added for 10 min. Absorbance at 450 nm was finally read with a spectrophotometer (iEMS, Labsystem, BMG Labtech Sarl, Champigny sur Marne, France).

Western blot analysis. After treatment, cells were lysed in RIPA buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1% (v/v) SDS, 0.5% (v/v) sodium deoxycholate, 100 μmol/L paramethylsulfonide, 1 μg/mL pepstatin, 2 μg/mL leupeptin] at 4°C and then boiled for 3 min. Proteins (50 μg) were separated on a polyacrylamide sodium dodecylsulfate–containing gel and transferred to a nitrocellulose membrane (Amersham, Orsay, France). After blocking nonspecific binding sites for 1 h at room temperature by 5% (w/v) skimmed milk in PBS with 0.1% (v/v) Tween 20 (TPBS), membranes were incubated for 2 h at room temperature or overnight at 4°C with the different antibodies. Membranes were then washed twice with TPBS and incubated for 1 h with peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies. Revelation was done by chemiluminescence.

ATP concentration measurement. ATP concentration was measured with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Charbonnières, France) according to the manufacturer's instructions. Briefly, after treatment, cells were lysed in the provided buffer and the luminescent substrate was added. Luminescence was measured with a fluorimeter/luminometer (SpectraMax Gemini XS). In parallel, cell viability was determined by a methylene blue colorimetric assay (25) in additional wells where cells have not been lysed but have been fixed in ethanol. ATP concentration was next expressed as percentage of ATP measured in untreated cells per cell number.

Measurement of intracellular superoxide anion by flow cytometry. DHE was used to detect intracellular superoxide anion production. After

[3] http://www.alexis-corp.com.

treatment, floating and adherent cells (0.5×10^6) were recovered and incubated in medium without FCS containing 5 μmol/L DHE for 15 min at 37°C. Dye oxidation (increase in FL-2 fluorescence) was measured using a FACScan flow cytometer (Becton Dickinson, Le Pont-De-Claix, France) with excitation and emission settings at 488 and 530 nm, respectively. A positive control was obtained by incubating cells with menadione (1 mmol/L). Superimposition of control and menadione histograms allowed to define a gate for calculating the percentage of cells producing superoxide anion.

Statistical analysis. Statistical analyses were carried out using the unilateral Student's *t* test considering the variances as unequal. The significance is shown as follows: *, $P \leq 0.05$; **, $P \leq 0.02$; and ***, $P \leq 0.01$.

Results

Cell death induced by TRAIL at acidic pH_e (6.5) exhibits morphologic and biochemical characteristics of programmed necrosis. After TRAIL treatment at acidic (6.5) or physiologic (7.4) pH_e, fluorescence microscopy of Hoechst/propidium iodide–stained cells was used to visualize the type of cell death (apoptosis or necrosis). At pH_e 7.4, blue typical apoptotic cells without plasma membrane breakdown were observed, showing chromatin condensation and nuclei shrunken or separated into spherical bodies (Fig. 1*A*). Under these conditions, HT29 cells were rather resistant to TRAIL because a 24-h TRAIL treatment induced ~20% of apoptosis (Fig. 1*B*). In contrast, at pH_e 6.5, HT29 cells became very sensitive to TRAIL and a 24-h TRAIL treatment induced ~80% of necrosis-like cell death characterized by red cells with the loss of plasma membrane integrity and the absence of chromatin fragmentation (Fig. 1*A* and *B*). We next did kinetics of TRAIL treatment. We observed that TRAIL-induced necrosis-like cell death occurred very rapidly and independently of apoptosis induction because 20% of red cells were detected without any detection of apoptotic cells after a 4-h TRAIL treatment at acidic pH_e (Fig. 1*B*). Moreover, TRAIL induced 80% of necrosis-like cell death without any apoptosis induction after a 24-h TRAIL treatment at pH_e 6.5 (Fig. 1*B*). TRAIL-induced cell death at acidic pH_e was also characterized by a high reactive oxygen species generation, with ~60% of positive cells stained with the DHE probe (Fig. 1*C*), and by ATP depletion (Fig. 1*D*), both of which representing characteristics of necrosis. In the first 8 h, intracellular ATP level quickly decreased in HT29 cells treated with TRAIL at acidic pH_e in comparison with low ATP depletion observed in HT29 cells treated with TRAIL at physiologic pH_e (Fig. 1*D*). To study if TRAIL-induced necrosis-like cell death at pH_e 6.5 was a reversible process, we did experiments where HT29 cells were treated with TRAIL at pH_e 6.5 during increased periods (2, 4, 6, and 8 h) before restoration of physiologic pH_e until 20 h. Under these experimental conditions, only restoration of pH_e to

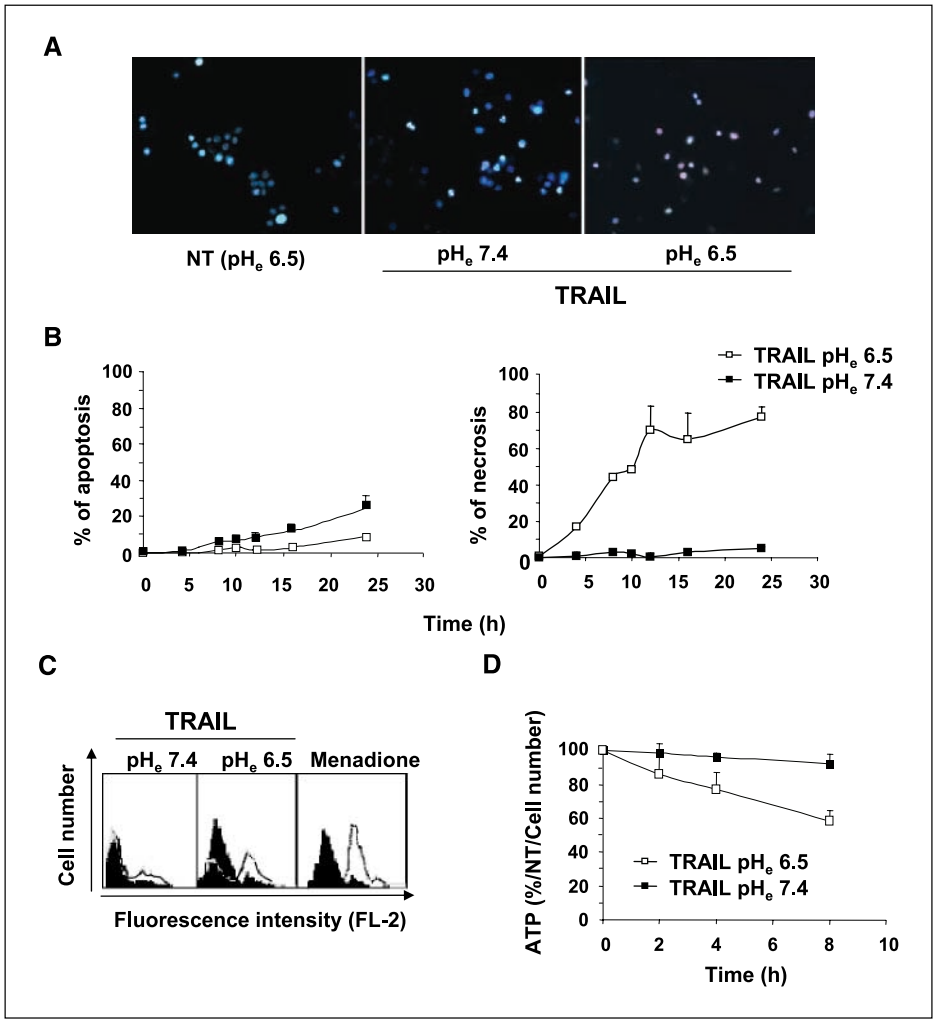

Figure 1. TRAIL induces cell death with features of necrosis at acidic pH_e. *A,* HT29 cells were treated with TRAIL (50 ng/mL cross-linked with 2 μg/mL anti-Flag M2) or not (*NT*) for 24 h at physiologic pH_e (7.4) or at acidic pH_e (6.5) and observed under fluorescence microscopy after Hoechst 33342 and propidium iodide costaining. *B,* HT29 cells were treated with TRAIL for the indicated times at physiologic pH_e (7.4) or at acidic pH_e (6.5). Percentages of apoptotic or necrotic cells were estimated by nuclear chromatin staining with Hoechst 33342 and propidium iodide. Percentage of apoptosis was measured as percentage of cells with fragmented condensed nucleus without propidium iodide staining. Percentage of necrosis-like cell death was estimated as cells with nonfragmented nucleus stained with propidium iodide. *Points,* mean ($n = 3$); *bars,* SD. *C,* superoxide anion production was studied by flow cytometry as described in Materials and Methods after treatment with TRAIL for 16 h at physiologic pH_e (7.4) or at acidic pH_e (6.5) and compared with untreated cells (*filled dark histograms*). One representative of three independent experiments. *D,* HT29 cells were treated with TRAIL at physiologic pH_e (7.4) or at acidic pH_e (6.5) for the indicated times. Intracellular ATP was measured as described in Materials and Methods. The values are expressed as percentage of untreated cells per cell number. *Points,* mean ($n = 3$); *bars,* SD.

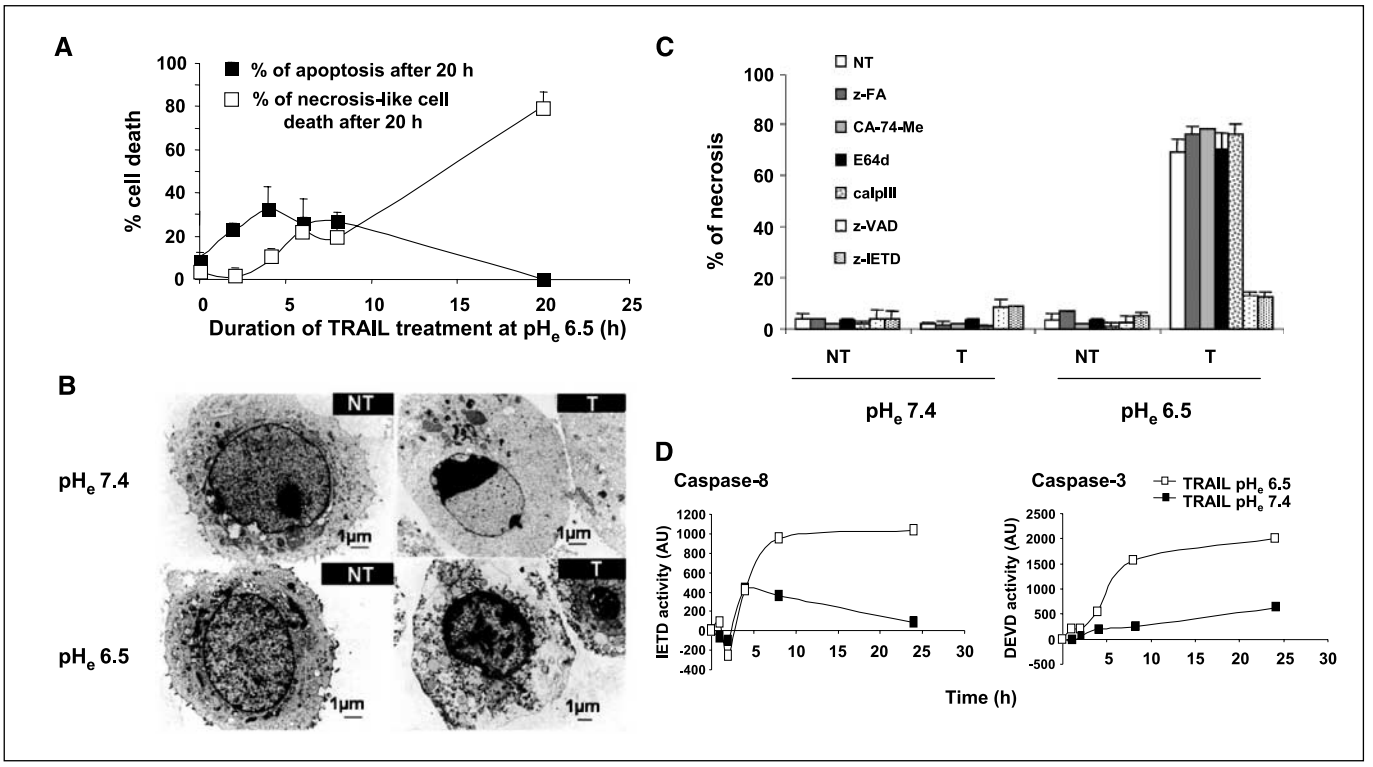

Figure 2. TRAIL-induced cell death at acidic pH_e is a reversible process and is dependent on caspase activation. *A,* HT29 cells were treated with TRAIL at acidic pH_e for the indicated times before restoration of physiologic pH_e by addition of sodium hydrogenocarbonate to reach a final concentration of 23 mmol/L. Percentages of apoptosis and necrosis-like cell death were estimated 20 h after TRAIL treatment at acidic pH_e. *Points,* mean (n = 3); *bars,* SD. *B,* electron microscopy analysis was done after a 24-h TRAIL treatment (*T*) or not (*NT*) at physiologic pH_e (7.4) or at acidic pH_e (6.5). *C,* HT29 cells were treated with TRAIL (50 ng/mL cross-linked with 2 μg/mL anti-Flag M2) or not at normal (pH_e 7.4) or acidic pH_e (6.5) for 24 h after a 1-h pretreatment or not with caspase inhibitor negative control z-FA-fmk (*z-FA*; 10 μmol/L), caspase-8 inhibitor z-IETD-fmk (*z-IETD*; 10 μmol/L), pan-caspase inhibitor z-VAD-fmk (*z-VAD*; 10 μmol/L), calpain inhibitor III (*calpIII*; 20 μmol/L), cathepsin B inhibitor (*CA-74-Me*; 25 μmol/L), or E64d (10 μmol/L). Percentage of necrosis was determined as in Fig. 1. *Columns,* mean (n = 3); *bars,* SD. *D,* caspase activation was measured in lysates from cell treated with TRAIL for the indicated times at physiologic pH_e (7.4) or at acidic pH_e (6.5) by using IETD-AFC (*Caspase-8*) or DEVD-AMC (*Caspase-3*) peptide substrates and expressed as arbitrary units (*AU*). One representative of three independent experiments.

physiologic value (7.4) during the first 4 h after TRAIL treatment at pH_e 6.5 restored TRAIL-induced apoptosis (Fig. 2*A*). Finally, to fully characterize the mode of TRAIL-induced cell death at acidic pH_e, we did an electron microscopic analysis. We observed that, after a 24-h TRAIL treatment at acidic pH_e, cells were swollen with increased cytoplasmic vacuolation, breakdown of plasma membrane and nuclear envelop, no or weak chromatin condensation, and no nuclear fragmentation (Fig. 2*B*). On the contrary, at physiologic pH_e, we clearly observed nuclear condensation and maintenance of plasma membrane integrity after a 24-h TRAIL treatment (Fig. 2*B*). All these data suggested that TRAIL induced a programmed necrosis-like cell death at acidic pH_e.

Caspases are activated and necessary for TRAIL-induced necrosis-like cell death at acidic pH_e (6.5). We have previously described that TRAIL-induced necrosis-like cell death at acidic pH_e was dependent on caspases (Fig. 2*C*; ref. 20). Because other proteases, such as calpain or cathepsins, could be involved in necrosis, we tested inhibitors of these proteases. None of these inhibitors of calpain or cathepsins was able to inhibit TRAIL-induced necrosis-like cell death at acidic pH_e or to induce cell death at physiologic pH_e (Fig. 2*C*). Furthermore, we observed that both initiator caspase-8 and effector caspase-3 were activated because IETD-AFC and DEVD-AMC, peptide substrates that mimic caspase-8 and caspase-3 target sites, respectively, were very rapidly cleaved on TRAIL treatment at acidic pH_e from 4 h until 24 h (Fig. 2*D*). In contrast, at pH_e 7.4, caspase-8 and caspase-3 activation was lower than at pH_e 6.5 (Fig. 2*D*). These results suggested that TRAIL induced a caspase-dependent necrosis-like cell death at pH_e 6.5.

TRAIL treatment induces RIP1 expression, which is not cleaved under acidic pH_e conditions. We next did kinetics of TRAIL treatment in HT29 cells under both physiologic and acidic pH_e conditions. Western blot analysis showed that the expression of RIP1 protein was increased in HT29 cells treated with TRAIL but more quickly at acidic pH_e (from 4 to 24 h) than at physiologic pH_e (from 8 to 24 h; Fig. 3*A*). Interestingly, RIP1 was cleaved on TRAIL treatment at pH_e 7.4 but not at pH_e 6.5 (Fig. 3*A*). The cleavage of RIP1 following TRAIL treatment at pH_e 7.4 was caspase-8 dependent because pretreatment with z-IETD-fmk, a caspase-8 inhibitor, prevented this cleavage (Fig. 3*B*).

TRAIL induced a necrosis-like cell death at pH_e 6.5 that depends on RIP1 kinase activity. Pretreatment with geldanamycin, a HSP90 function inhibitor, reverted TRAIL-induced necrosis-like cell death to apoptosis at acidic pH_e (Fig. 4*A*). As it has been shown that disruption of HSP90 function by addition of geldanamycin results in degradation of RIP1, we studied by Western blot analysis the expression of RIP1 following TRAIL treatment under both physiologic and acidic pH_e conditions in HT29 cells. Concomitant with the inhibition of TRAIL-induced necrosis-like cell death by geldanamycin, RIP1 expression was

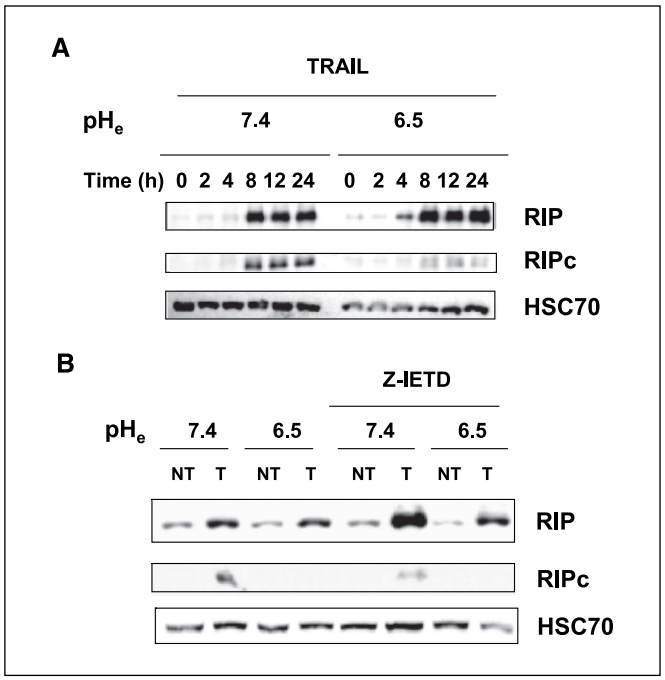

Figure 3. TRAIL induces RIP expression at both pH_e and RIP cleavage only at physiologic pH_e. *A,* HT29 cells were treated with TRAIL (50 ng/mL cross-linked with 2 μg/mL anti-Flag M2) for the indicated times at physiologic pH_e (7.4) or at acidic pH_e (6.5), and Western blot analysis of RIP expression was done as described in Materials and Methods. Anti-human HSC70 was used as a control of protein loading. One representative of three independent experiments. *B,* HT29 cells were treated or not with TRAIL for 24 h after a 1-h pretreatment or not with 10 μmol/L z-IETD-fmk. Western blot analysis of RIP expression was done as described in Materials and Methods. Anti-human HSC70 was used as a control of protein loading. One representative of three independent experiments.

highly decreased in TRAIL-treated HT29 cells after geldanamycin pretreatment (Fig. 4*B*). Consistent with the observation that geldanamycin pretreatment led to a switch from TRAIL-induced necrosis-like cell death to apoptotic cell death at acidic pH_e (Fig. 4*A*), PARP cleavage was restored under these conditions (Fig. 4*B*). The quantitative analysis of PARP cleavage (band at 85 kDa) showed that the cleavage of PARP was detected in HT29 cells treated with TRAIL at pH_e 7.4 after or not a geldanamycin pretreatment and only in HT29 cells treated with TRAIL at pH_e 6.5 after a geldanamycin pretreatment when apoptosis was restored (Fig. 4*A* and *B*). We next did RNA interference experiments with RIP1 small interfering RNAs (siRNA). We observed that transfection with RIP1 siRNAs completely reduced RIP protein expression in HT29 cells (Fig. 5*A*) and induced a significant (~30 %) inhibition of TRAIL-induced necrosis-like cell death at pH_e 6.5 (Fig. 5*B*). This transfection did not modify the low level of necrosis induced by TRAIL at pH_e 7.4 (Fig. 5*B*). On the contrary, transfection with nonspecific siRNAs had effect neither on cell death induction nor on RIP protein expression (Fig. 5*A* and *B*). We further did transient transfection with both kinase-dead RIP-expressing vectors (RIP K45R and RIP D138N) and RIP WT to evaluate TRAIL-induced necrosis under acidic conditions. These experiments showed that the functional RIP kinase domain was necessary for TRAIL-induced necrosis because expression of the kinase-dead RIP constructs induced ~60% inhibition compared with the WT construct (Fig. 5*C*).

TRAIL-induced NF-κB activation at acidic pH_e (6.5) is not essential for necrosis-like cell death induction. As RIP1 has been involved in NF-κB activation (15, 16, 26), we used an ELISA assay based on p65 binding activity to specific consensus DNA sequence to detect NF-κB activation following TRAIL treatment at both physiologic and acidic pH_e. This assay showed that NF-κB was activated in HT29 cells treated with TRAIL but much more efficiently at acidic pH_e than at physiologic pH_e (Fig. 6*A*). Consistent with these data, we observed a transient IκB-α phosphorylation at both physiologic and acidic pH_e from 2 to 4 h on TRAIL treatment (Fig. 6*B*). Furthermore, IκB-α was degraded in HT29 cells treated with TRAIL at acidic pH_e (Fig. 6*B*). TRAIL-induced NF-κB activation at pH_e 6.5 was inhibited by geldanamycin pretreatment, suggesting the implication of RIP1 in this activation (Fig. 6*C*). Interestingly, pretreatment with z-IETD-fmk, a caspase-8 inhibitor, had no effect on TRAIL-induced NF-κB activation at pH_e 6.5 (Fig. 6*C*), whereas it inhibits TRAIL-induced necrosis-like cell death at pH_e 6.5 (Fig. 2*C*), suggesting that NF-κB activation was dispensable for TRAIL-induced necrosis at pH_e 6.5. This was confirmed by using Bay 117085, a specific NF-κB inhibitor. Indeed, pretreatment with this inhibitor had no effect on TRAIL-induced necrosis-like cell death at acidic pH_e (Fig. 6*D*). Altogether, these data suggest that NF-κB signaling is not essential to TRAIL-induced necrosis at pH_e.

Discussion

We have previously described that, under acidic pH_e (6.5) conditions, TRAIL induced a necrosis-like cell death in human HT29 colon cancer and HepG2 hepatocarcinoma cell lines. This cell death seemed to be dependent on TRAIL-R1/TRAIL-R2 death receptors and caspases and was partially inhibited by Bcl-2 expression (20). We investigated here the molecular mechanisms involved in this caspase-dependent necrosis-like cell death. We observed by electron microscopy analysis that, at acidic pH_e, TRAIL-treated HT29 cells presented an enlarged cytoplasm, breakdown of plasma membrane, and nuclear envelop with chromatin condensation in some extent but no nuclear fragmentation in contrast to what was observed at physiologic pH_e. These morphologic characteristics of necrotic cells (27) further confirmed that, at acidic pH_e, TRAIL induced a necrosis-like PCD in HT29 cells. Whereas TRAIL induced a caspase-independent necrosis in TRAMP-C2 murine prostate adenocarcinoma cells (13), TRAIL induced a caspase-dependent necrosis-like cell death at pH_e 6.5 in HT29 human colon cancer cells because pretreatment with caspase inhibitors z-VAD-fmk or z-IETD-fmk completely inhibited this form of cell death (20). This observation suggested that TRAIL-induced necrosis-like cell death at pH_e 6.5 required caspase activation as well as the TRAIL apoptotic death pathway. Activation of caspases was confirmed by measuring the released fluorescence after the cleavage of fluorescent peptide substrates mimicking caspase-8 and caspase-3 target sites. Caspase-8 and caspase-3 activations were observed to be greater in HT29 cells after TRAIL treatment at acidic pH_e (6.5) than at physiologic pH_e (7.4). The very high level of caspase activation in TRAIL-treated HT29 cells at acidic pH_e (6.5) might be accounted for cytosolic acidification (20). Indeed, several caspases are targets for cytosolic acidification and their activation is facilitated by acidic pH_e (23). All these data are in accordance with some reports describing caspase-dependent FasL-induced necrosis (28) or other caspase-dependent necrosis-like cell death pathways (29, 30).

We also did kinetics to study RIP1 expression in HT29 cells after TRAIL treatment under both physiologic and acidic pH_e conditions. We showed that RIP1 expression levels were very low in HT29 cells but highly increased on TRAIL treatment at both acidic or physiologic pH_e. However, the kinetics of RIP1 induction in HT29 cells were faster after TRAIL treatment at acidic (from 4 to 24 h) compared with physiologic pH_e (from 8 to 24 h). Induction of RIP has already been described in lipopolysaccharide-treated lymphocytes (19) or oltipraz-treated HT29 and HCT-116 cells (31). However, the molecular mechanisms of such an induction remain unknown. It has also been previously described that RIP1 could be cleaved by caspase-8 and that the COOH-terminal cleavage product of RIP1 was an inducer of apoptosis and an inhibitor of NF-κB signaling (32). Here, we observed that a caspase-8-dependent cleavage product of RIP1 was generated during stimulation by TRAIL at pH_e 7.4, which correlated with the induction of apoptosis and with a low level of NF-κB activation in HT29 cells. In contrast, RIP1 was not cleaved after TRAIL treatment at pH_e 6.5, a finding that correlated with the induction of necrosis and the strong activation of NF-κB in HT29 cells. These data therefore suggested that TRAIL-induced necrosis-like cell death at pH_e 6.5 might depend on RIP1 expression. To test this hypothesis, we used geldanamycin, known to induce degradation of RIP1 protein (17). Using geldanamycin, we could show that RIP1-induced degradation reversed TRAIL-induced necrosis-like cell death to apoptosis at acidic pH_e. Accordingly, the use of RIP1 siRNAs by transient transfection inhibited TRAIL-induced necrosis-like cell death at pH_e 6.5. Moreover, transient transfections of RIP kinase-dead mutants using plasmids encoding either a mutant in which the conserved lysine in the kinase subdomain II was mutated (RIP K45R) or which has been mutated within the phosphotransferase loop of subdomain VI (RIP D138N) strongly inhibited TRAIL-induced necrosis-like cell death. Both mutants were shown to lack the kinase activity (33) and to also inhibit Fas-induced necrosis (10). Because HT29 cells exhibit very low endogenous RIP protein level, our results suggest that RIP kinase-dead mutant expression has behaved as a dominant-negative protein. It has recently been shown that RIP is necessary for disruption of the interaction between cyclophilin D and anion nucleotide translocator leading to a decrease in ATP and subsequently to necrosis (34). However, the implication of RIP kinase activity in this pathway has not yet been determined. We have also observed a decrease in ATP concentration following TRAIL treatment under acidic extracellular conditions, and because TRAIL-induced necrosis can partially be inhibited by Bcl-2 expression (20), our findings emphasize the possibility that RIP might target the mitochondria. However, the target of RIP kinase activity has not thus far been identified.

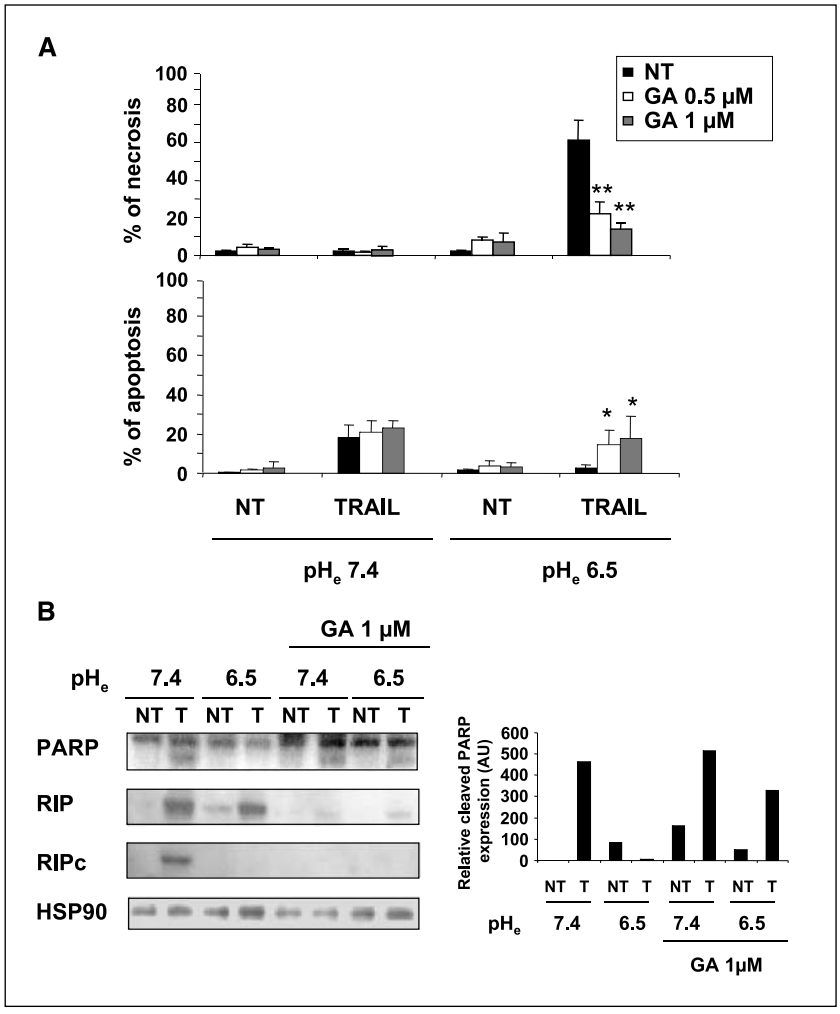

Figure 4. Geldanamycin pretreatment inhibited TRAIL-induced necrosis and restored TRAIL-induced apoptosis with PARP cleavage at acidic pH_e. *A,* HT29 cells were treated with TRAIL (50 ng/mL cross-linked with 2 μg/mL anti-Flag M2) or not for 24 h at physiologic pH_e (7.4) or at acidic pH_e (6.5) after a 1-h pretreatment or not with geldanamycin (*GA*; 0.5 or 1 μmol/L). Percentage of necrosis-like or apoptotic cell death was estimated as in Fig. 1. *Columns,* mean (n = 3); *bars,* SD. *B,* HT29 cells were treated or not for 24 h with TRAIL after a 1-h pretreatment or not with geldanamycin (1 μmol/L), and Western blot analysis of PARP and RIP expression was done as described in Materials and Methods. The relative cleaved PARP expression was expressed in arbitrary units as percentage of absorbance measured in untreated cells. Anti-human HSP90 was used as a control of protein loading. One representative of three independent experiments.

Surprisingly, RIP1 was not cleaved when the level of caspase-8 activation was very high, suggesting either the subcellular compartmentalization of caspase-8 or RIP1 or the inaccessibility of the substrate RIP1. However, fractionation of HT29 cell lysates in subcellular fractions (cytosolic, membrane, nuclear, and cytoskeletal) after TRAIL treatment at acidic or physiologic pH_e did not show any particular subcellular compartmentalization of caspase-8 or RIP1 at pH_e 6.5 (data not shown). Modification of RIP1 rendering the caspase cleavage site inaccessible might be another possibility; about that point, it is worth noting that phosphorylation of serine residues adjacent to the caspase-3 cleavage site of presinilin-2 has been reported to protect the protein from cleavage (35). However, until now, RIP1 kinase has been shown to autophosphorylate itself at serine/threonine residues, which remains to be identified (36). Moreover, selective processing of substrates by caspase-8 at pH_e 6.5 could also account for the absence of RIP1 cleavage similarly to what was previously described following T-cell receptor stimulation (37). It is interesting to notice that, whereas RIP was not cleaved at pH_e 6.5 on TRAIL treatment, Bid was cleaved in its truncated form (data not shown). This cleavage of Bid could account, at least in part, for the implication of caspase activation in this necrotic pathway.

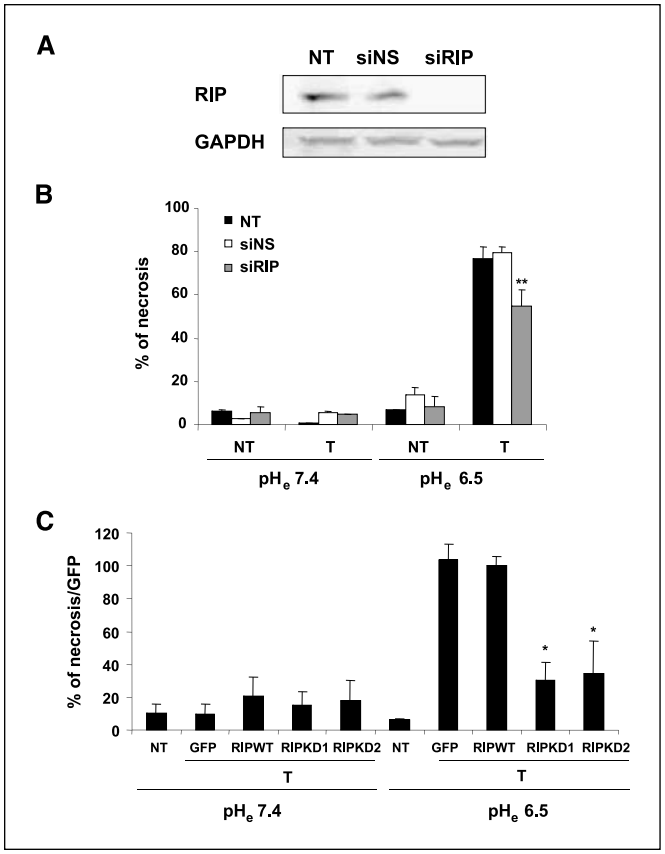

Figure 5. RIP and its kinase activity are necessary for TRAIL-induced necrosis-like cell death at acidic pH_e. *A,* HT29 cells transfected or not with a scramble nonspecific small interference RNA (*siNS*) or RIP1 small interference RNA (*siRIP*) using TransFectin lipid reagent as described in Materials and Methods. Western blot analysis of RIP expression was carried out as described in Materials and Methods 48 h after transfection. As endogenous RIP expression level was weak in HT29 cells, Western blot was overexposed. Anti-human GAPDH was used as a control of protein loading. One representative of three independent experiments. *B,* HT29 cells transfected or not with a scramble nonspecific small interference RNA or RIP1 small interference RNA using TransFectin lipid reagent as above were treated or not with TRAIL for 24 h at physiologic pH_e (7.4) or at acidic pH_e (6.5). Percentage of necrosis was estimated as described in Fig. 1. *Columns,* mean (n = 6); *bars,* SD. *C,* HT29 cells transfected or not using Amaxa nucleofector as described in Materials and Methods with 3 μg pmaxGFP alone (*GFP*) or in combination with 3 μg pMSCV-RIP-WT (*RIPWT*) or pMSCV-RIP.K45R (*RIPKD1*) or pMSCV-RIP.D138N (*RIPKD2*) were treated or not with TRAIL for 24 h at physiologic pH_e (7.4) or at acidic pH_e (6.5). Necrotic cell death was then analyzed by flow cytometry, and the percentage of necrosis was determined as the percentage of propidium iodide–positive cells among GFP-positive cells. *Columns,* mean (n = 3); *bars,* SD.

In some cellular systems, necrosis-like cell death has already been reported to occur downstream of mitochondria (38). Our data suggest that TRAIL-induced necrosis-like cell death at pH_e 6.5 could occur upstream of mitochondria at the level of caspase-8 because z-IETD-fmk, a caspase-8 inhibitor, completely blocked TRAIL-induced necrosis-like cell death at pH_e 6.5, whereas Bcl-2 expression in HT29 cells partially inhibited this cell death (20).

TRAIL is usually described as an apoptosis inducer. A recent study has shown that TRAIL could induce a caspase-independent necrosis-like cell death in TRAMP-C2 murine prostate adenocarcinoma cells (13). We showed for the first time that TRAIL induced RIP1- and caspase-dependent necrosis-like cell death in HT29 cells at acidic pH_e. It has also been shown that death receptor could induce necrosis-like cell death under certain circumstances (1, 10–12). Nevertheless, necrosis-like PCD signaling is still poorly understood. We observed here that RIP1 is necessary for necrosis-like cell death induced by TRAIL under acidic extracellular conditions. Thus, the apoptosis/necrosis switch under acidic pH_e condition is an early decision in the signaling of cell death because it was possible to revert the necrotic signaling pathway by restoring a physiologic pH_e only until 4 h after TRAIL treatment at acidic pH_e.

As RIP1 is also essential for NF-κB activation, we have studied NF-κB activation after TRAIL treatment at acidic and physiologic pH_e as well as its involvement in TRAIL-induced necrosis-like cell death at pH_e 6.5. We showed here that the level of NF-κB activation was very high in HT29 cells treated with TRAIL at pH_e 6.5 and was dependent on RIP1 expression because geldanamycin pretreatment completely inhibited NF-κB activation. However, NF-κB activation was dispensable for TRAIL-induced necrosis-like cell death at pH_e 6.5. Moreover, z-IETD-fmk, an inhibitor of caspase-8, which inhibited TRAIL-induced necrosis-like cell death at pH_e 6.5, did not inhibit NF-κB activation, suggesting that activation of NF-κB was not mediated by caspase-8 unlike what has been reported in other cell systems (26, 39). We also observed that, at pH_e 6.5, TRAIL treatment induced IL-8 production (data not shown). As IL-8 production is usually associated with inflammation, it is interesting to notice that necrosis induced by TRAIL at acidic pH_e could happen in a proinflammatory context, which could be beneficial for stimulation of an antitumor immune response (2). It has also been proposed that cell death through necrosis could be more immunogenic than cell death through apoptosis (40).

As pH_e of solid tumors is in most cases acidic (22), this new TRAIL death pathway may have an important effect on TRAIL efficiency in cancer therapy. We tested in this study an pH_e value that can be observed *in vivo*. HT29 colon tumors xenografted in mice exhibit an acidic extracellular environment 3 or 4 weeks after cell injection (41) that could make it possible for TRAIL to induce a necrosis-like cell death *in vivo*. However, Ashkenazi et al. (42) have reported a typical apoptosis with PARP cleavage in HCT-116 human colon tumors xenografted in mice after TRAIL treatment. It is important to note that TRAIL

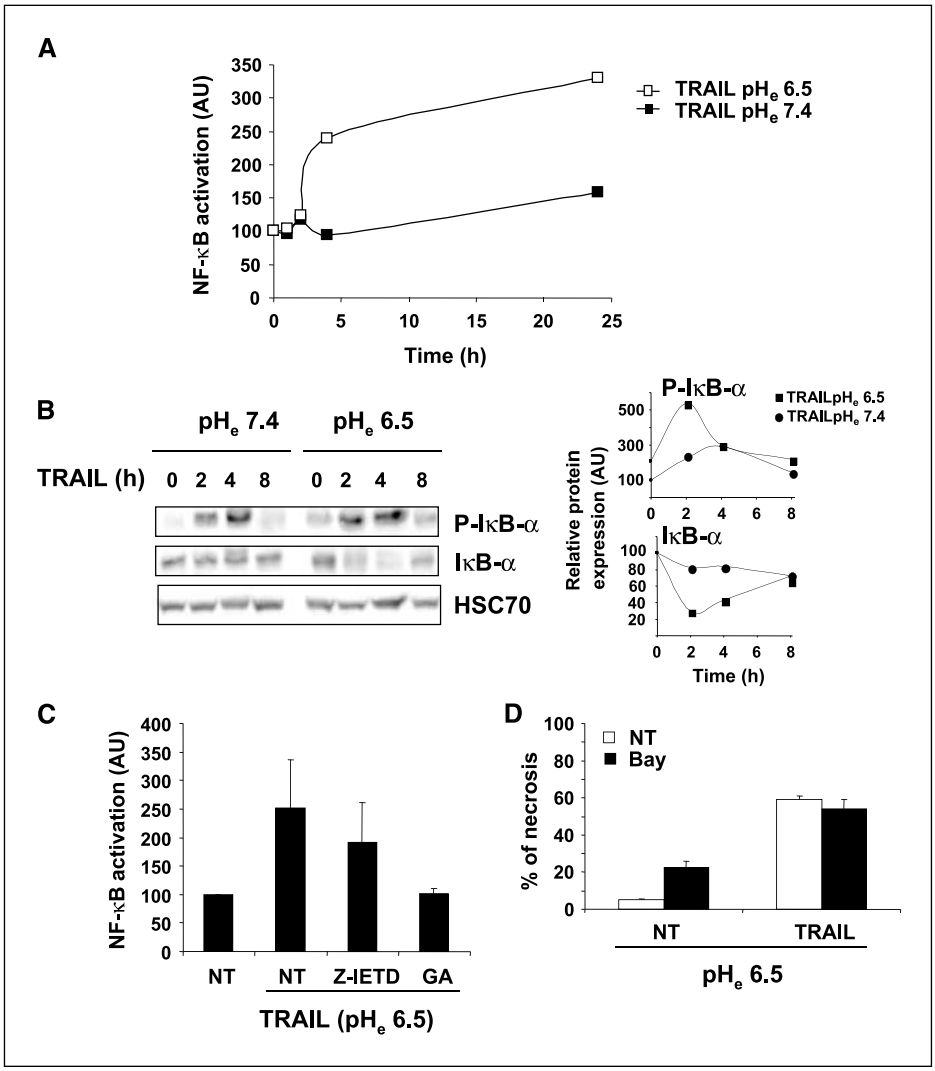

Figure 6. NF-κB is activated but dispensable for TRAIL-induced necrosis-like cell death at acidic pH_e. *A,* HT29 cells were treated with TRAIL (50 ng/mL cross-linked with 2 μg/mL anti-Flag M2) for the indicated times at physiologic pH_e (7.4) or at acidic pH_e (6.5). p65 DNA binding activity was estimated from nuclear extracts as described in Materials and Methods and expressed in arbitrary units as percentage of activity detected in untreated cells (time 0). *B,* HT29 cells were treated with TRAIL for the indicated times at physiologic pH_e (7.4) or at acidic pH_e (6.5). Western blot analysis of phosphorylated IκB-α and IκB-α expression was done as described in Materials and Methods. Relative phosphorylated IκB-α (*P-IκB-α*) and IκB-α protein expressions were expressed in arbitrary units as percentage of absorbance measured in untreated cells. Anti-human HSC70 was used as a control of protein loading. One representative of three independent experiments. *C,* HT29 cells were treated with TRAIL or not at acidic pH_e (6.5) for 24 h after a 1-h pretreatment or not with 10 μmol/L z-IETD-fmk or with 1 μmol/L geldanamycin. p65 DNA binding activity was estimated as described above. *Columns,* mean (n = 3); *bars,* SD. *D,* HT29 cells were treated with TRAIL or not at acidic pH_e (6.5) after a 1-h pretreatment or not with 10 μmol/L Bay 117085 (*Bay*). Percentage of necrosis was estimated as in Fig. 1. *Columns,* mean (n = 3); *bars,* SD.

treatment began only 10 days after s.c. injection of HCT-116 cells, which could explain that, in this study, TRAIL mainly triggered an apoptotic pathway in HCT-116-derived tumors. Nevertheless, the relevance of this new cell death pathway induced by TRAIL for cancer therapy requires further *in vivo* investigations. In conclusion, our data suggest that TRAIL would be more efficient for the treatment of solid tumors exhibiting an acidic pH_e.

Acknowledgments

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References

1. Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 1997;185:1481–6.
2. Proskuryakov SY, Konoplyannikov AG, Gabai VL. Necrosis: a specific form of programmed cell death? Exp Cell Res 2003;283:1–16.
3. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 2004; 18:1272–82.
4. Borst P, Rottenberg S. Cancer cell death by programmed necrosis? Drug Resist Updat 2004;7:321–4.
5. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305–8.
6. Laster SM, Wood JG, Gooding LR. Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. J Immunol 1988;141:2629–34.
7. Fiers W, Beyaert R, Declercq W, Vandenabeele P. More than one way to die: apoptosis, necrosis, and reactive oxygen damage. Oncogene 1999;18:7719–30.
8. Wilson CA, Browning JL. Death of HT29 adenocarcinoma cells induced by TNF family receptor activation is caspase-independent and displays features of both apoptosis and necrosis. Cell Death Differ 2002;9: 1321–33.
9. Eguchi Y, Shimizu S, Tsujimoto Y. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res 1997;57:1835–40.
10. Holler N, Zaru R, Micheau O, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000;1:489–95.
11. Vercammen D, Beyaert R, Denecker G, et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 1998;187:1477–85.

12. Matsumura H, Shimizu Y, Ohsawa Y, Kawahara A, Uchiyama Y, Nagata S. Necrotic death pathway in Fas receptor signaling. J Cell Biol 2000;151:1247–56.

13. Kemp TJ, Kim JS, Crist SA, Griffith TS. Induction of necrotic tumor cell death by TRAIL/Apo-2L. Apoptosis 2003;8:587–99.

14. Stanger BZ, Leder P, Lee TH, Kim E, Seed B. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell 1995;81:513–23.

15. Hur GM, Lewis J, Yang Q, et al. The death domain kinase RIP has an essential role in DNA damage-induced NF-κB activation. Genes Dev 2003;17:873–82.

16. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-κB signal. Immunity 1998;8:297–303.

17. Vanden Berghe T, Kalai M, van Loo G, Declercq W, Vandenabeele P. Disruption of HSP90 function reverts tumor necrosis factor-induced necrosis to apoptosis. J Biol Chem 2003;278:5622–9.

18. Shen HM, Lin Y, Choksi S, et al. Essential roles of receptor-interacting protein and TRAF2 in oxidative stress-induced cell death. Mol Cell Biol 2004;24:5914–22.

19. Meylan E, Tschopp J. The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci 2005; 30:151–9.

20. Meurette O, Huc L, Rebillard A, Le Moigne G, Lagadic-Gossmann D, Dimanche-Boitrel MT. TRAIL (TNF-related apoptosis-inducing ligand) induces necrosis-like cell death in tumor cells at acidic extracellular pH. Ann N Y Acad Sci 2005;1056:379–87.

21. Wike-Hooley JL, van den Berg AP, van der Zee J, Reinhold HS. Human tumour pH and its variation. Eur J Cancer Clin Oncol 1985;21:785–91.

22. Stubbs M, McSheehy PM, Griffiths JR, Bashford CL. Causes and consequences of tumour acidity and implications for treatment. Mol Med Today 2000;6:15–9.

23. Lagadic-Gossmann D, Huc L, Lecureur V. Alterations of intracellular pH homeostasis in apoptosis: origins and roles. Cell Death Differ 2004;9:953–61.

24. Lee YJ, Song JJ, Kim JH, Kim HR, Song YK. Low extracellular pH augments TRAIL-induced apoptotic death through the mitochondria-mediated caspase signal transduction pathway. Exp Cell Res 2004;293: 129–43.

25. Micheau O, Solary E, Hammann A, Martin F, Dimanche-Boitrel MT. Sensitization of cancer cells treated with cytotoxic drugs to fas-mediated cytotoxicity. J Natl Cancer Inst 1997;89:783–9.

26. Kreuz S, Siegmund D, Rumpf JJ, et al. NFκB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. J Cell Biol 2004;166:369–80.

27. Ziegler U, Groscurth P. Morphological features of cell death. News Physiol Sci 2004;19:124–8.

28. Leist M, Nicotera P. Apoptosis versus necrosis: the shape of neuronal cell death. Results Probl Cell Differ 1998;24:105–35.

29. Schwab BL, Guerini D, Didszun C, et al. Cleavage of plasma membrane calcium pumps by caspases: a link between apoptosis and necrosis. Cell Death Differ 2002; 9:818–31.

30. Wang X, Ryter SW, Dai C, et al. Necrotic cell death in response to oxidant stress involves the activation of the apoptogenic caspase-8/bid pathway. J Biol Chem 2003; 278:29184–91.

31. Nho CW, O'Dwyer PJ. NF-κB activation by the chemopreventive dithiolethione oltipraz is exerted through stimulation of MEKK3 signaling. J Biol Chem 2004;279:26019–27.

32. Kim JW, Choi EJ, Joe CO. Activation of death-inducing signaling complex (DISC) by pro-apoptotic C-terminal fragment of RIP. Oncogene 2000;19:4491–9.

33. Ting AT, Pimentel-Muinos FX, Seed B. RIP mediates tumor necrosis factor receptor 1 activation of NF-κB but not Fas/APO-1-initiated apoptosis. EMBO J 1996;15: 6189–96.

34. Temkin V, Huang Q, Liu H, Osada H, Pope RM. Inhibition of ADP/ATP exchange in receptor-interacting protein-mediated necrosis. Mol Cell Biol 2006;26: 2215–25.

35. Walter J, Schindzielorz A, Grunberg J, Haass C. Phosphorylation of presenilin-2 regulates its cleavage by caspases and retards progression of apoptosis. Proc Natl Acad Sci U S A 1999;96:1391–6.

36. Sun X, Yin J, Starovasnik MA, Fairbrother WJ, Dixit VM. Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. J Biol Chem 2002;277: 9505–11.

37. Alam A, Cohen LY, Aouad S, Sekaly RP. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. J Exp Med 1999;190:1879–90.

38. Hirsch T, Marchetti P, Susin SA, et al. The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. Oncogene 1997;15:1573–81.

39. Su H, Bidere N, Zheng L, et al. Requirement for caspase-8 in NF-κB activation by antigen receptor. Science 2005;307:1465–8.

40. Melcher A, Gough M, Todryk S, Vile R. Apoptosis or necrosis for tumor immunotherapy: what's in a name? J Mol Med 1999;77:824–33.

41. Ojugo AS, McSheehy PM, McIntyre DJ, et al. Measurement of the extracellular pH of solid tumours in mice by magnetic resonance spectroscopy: a comparison of exogenous (19)F and (31)P probes. NMR Biomed 1999;12: 495–504.

42. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155–62.