Rottlerin Sensitizes Colon Carcinoma Cells to Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis via Uncoupling of the Mitochondria Independent of Protein Kinase C

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ABSTRACT

Signaling pathways involved in survival responses may attenuate the apoptotic response to the cytotoxic tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in human colon carcinomas. In six lines examined, three were sensitive (GC/c1, VRC/c1, HCT116), HT29 demonstrated intermediate sensitivity, and RKO and HCT8 were resistant to TRAIL-induced apoptosis. Calphostin c [an inhibitor of classic and novel isoforms of protein kinase C (PKC)] sensitized five of six cell lines to TRAIL, whereas Go6976, (inhibitor of classic PKC isoforms), did not influence TRAIL sensitivity. Rottlerin, an inhibitor of novel isoforms of PKC, specifically PKCδ, sensitized five of six cell lines to TRAIL-induced apoptosis, suggesting that PKCδ may be involved in the mechanism of TRAIL resistance. Transfection of HCT116 with a proapoptotic cleaved fragment of PKCδ or an antiapoptotic full-length PKCδ did not influence the sensitivity of HCT116 to TRAIL. Furthermore, the incubation of HCT116 or RKO with phorbol myristate acetate for 16 h, which downregulated the expression of novel PKC isoforms, also did not influence sensitivity to TRAIL either in the absence or presence of rottlerin. However, after 15-min incubation with rottlerin, mitochondrial membrane potential (ΔΨm) was dramatically reduced in RKO cells, and, in cells subsequently treated with TRAIL, rapid apoptosis was evident within 8 h. Calphostin c, but not Go6976, also caused a decrease in ΔΨm. In RKO, rottlerin induced the release of cytochrome c, HtrA2/Omi, Smac/DIABLO, and AIF from the mitochondria, potentiated in combination with TRAIL, with concomitant caspase activation and down-regulation of XIAP. In HT29, the release of proapoptotic factors was demonstrated only when rottlerin and TRAIL were combined, and Bcl-2 overexpression inhibited this release and the induction of apoptosis. TRAIL-induced apoptosis was not influenced by rottlerin or Bcl-2 overexpression in type I (GC/c1) cells. Data suggest that rottlerin affects mitochondrial function independent of PKCδ, thereby sensitizing cells to TRAIL, and that mitochondria constitute an important target in overcoming inherent resistance to TRAIL in colon carcinomas.

INTRODUCTION

TRAIL, a type II transmembrane protein, is a cytotoxic ligand belonging to the TNF family of ligand and receptor pairs, which modulate the expression of novel PKC isoforms, also did not influence sensitivity to TRAIL either in the absence or presence of rottlerin. However, after 15-min incubation with rottlerin, mitochondrial membrane potential (ΔΨm) was dramatically reduced in RKO cells, and, in cells subsequently treated with TRAIL, rapid apoptosis was evident within 8 h. Calphostin c, but not Go6976, also caused a decrease in ΔΨm. In RKO, rottlerin induced the release of cytochrome c, HtrA2/Omi, Smac/DIABLO, and AIF from the mitochondria, potentiated in combination with TRAIL, with concomitant caspase activation and down-regulation of XIAP. In HT29, the release of proapoptotic factors was demonstrated only when rottlerin and TRAIL were combined, and Bcl-2 overexpression inhibited this release and the induction of apoptosis. TRAIL-induced apoptosis was not influenced by rottlerin or Bcl-2 overexpression in type I (GC/c1) cells. Data suggest that rottlerin affects mitochondrial function independent of PKCδ, thereby sensitizing cells to TRAIL, and that mitochondria constitute an important target in overcoming inherent resistance to TRAIL in colon carcinomas.

TRAIL, a type II transmembrane protein, is a cytotoxic ligand belonging to the TNF family of ligand and receptor pairs, which include FasL/Fas and TNF/TNFFR1. TRAIL binds to four membrane-bound receptors (DR4, DR5, DcR1, DcR2). DR4 and DR5 contain a functional death domain and transmit an apoptotic signal via their 80-amino-acid intracellular death domains (1–3), which recruit adaptor proteins. However DcR1, which lacks an intracellular domain (4, 5), and DcR2, containing a truncated death domain (6, 7), constitute decoy receptors that sequester the ligand but are incapable of initiating an apoptotic signal. Unlike FasL and TNF, which have restricted tissue distribution, TRAIL is constitutively expressed in a wide variety of cells and tissues (8, 9). However few normal cell populations are sensitive to TRAIL (10, 11), in contrast to diverse types of malignant and transformed cell lines that are highly sensitive to the ligand (11). Although the TRAIL signaling pathway(s) remain to be fully elucidated, the receptor complexes formed after ligation of TRAIL are closer in composition to complexes formed with Fas (11, 12) than with TNFR1 (11). After ligation of TRAIL to DR4 or DR5 and trimerization of the receptor, FADD is recruited to the DISC followed by procaspase-8 (13–15). Death receptor signaling is considered to occur either via a type I pathway involving activation of large amounts of caspase-8 at the DISC followed by direct activation of downstream effector caspases, or via a type II pathway that requires mitochondrial involvement leading to caspase activation and a feedback amplification loop in the induction of apoptosis (16).

TRAIL resistance in malignant cells has not generally correlated with the relative levels of expression of DR4 and DR5 or the decoy receptors (17), suggesting the involvement of alternate mechanisms. PKC isoforms have been implicated in the mechanism of attenuation of death receptor-induced apoptosis (18–27). PKC constitutes a family of serine-threonine kinases comprising 12 different isoforms, which have been classified into three major groups based on their structures and on their activation mechanisms: conventional (α, β, γ), novel (δ, ε, η, θ), and atypical (ζ, χ, λ Ref. 19). PKCs play critical roles in cell proliferation, differentiation, neoplastic transformation, and apoptosis. The classic α, β, (18), novel δ (20–24), ε (19, 25), or θ (25, 26) and atypical ζ (27) PKC isoforms have demonstrated a role in the regulation of death receptor-induced apoptosis. Activation of PKC by phorbol esters has delayed Fas-mediated apoptosis (28), whereas the inhibition of PKC has enhanced Fas-induced (28, 29) and TRAIL-induced (19) cell death. A requirement for the induction of apoptosis in the presence of PKC (PKCδ or PKCθ) appears to be cleavage of the protein to a catalytically active fragment, mediated by caspase-3 (20, 26).

In the present investigation, we have demonstrated that inhibitors of novel isoforms of PKC sensitized human colon carcinoma cell lines to TRAIL-induced apoptosis. In particular, rottlerin, which has demonstrated selective action in the inhibition of PKCδ activity (30–34), was highly effective in sensitizing cell lines to TRAIL, suggesting that PKCδ may play a critical role in attenuating TRAIL-induced apoptosis. However, studies demonstrated that the modulation of PKCδ, either by genetic regulation or by PMA treatment, did not influence TRAIL-induced apoptosis, indicating that the mechanism of rottlerin action was independent of PKCδ. More recently, rottlerin has been found to act in a PKCδ-independent manner by acting as an uncoupler of oxidative phosphorylation, thereby disrupting the ΔΨm (35, 36) in a manner similar to classic mitochondrial uncouplers such as carbonyl cyanide p-trifluoromethoxyphenylydrazene (FCCP; Ref. 35). We subsequently demonstrated in human colon carcinoma cell lines that rottlerin induced a significant loss in ΔΨm within 15 min, and accel-
erated the onset of TRAIL-induced apoptosis in TRAIL-resistant lines. These data demonstrate that rottlerin disrupts mitochondrial function independent of PKC and suggest that the mitochondria may constitute an important target in the sensitization of human colon carcinoma cells to TRAIL.

MATERIALS AND METHODS

Cell Lines. The HT29, HCT8, and HCT116 human colon carcinoma cell lines were obtained from American Type Culture Collection. GC/c1 and VRC/c1 were established as reported previously (37), and RKO was obtained from Dr. Michael Kastan, St. Jude Children’s Research Hospital, Memphis, TN. Cells were maintained in the presence of folate-free RPMI 1640 containing 10% dFBS and 80 μM 5-methyltetrahydrofolate.

Production of Recombinant Human TRAIL. The cDNA of the extracellular domain of TRAIL corresponding to amino acids 114–281 was subcloned into the pET17b (Novagen) bacterial expression vector and expressed in the BL21(DE3)pLYS3 (Novagen) bacterial host. After induction of TRAIL expression using isopropyl-β-thio-galactosidase (IPTG; 1 mm), bacterial pellets were harvested, and TRAIL was purified after passage through a nickel column (Ni-NTA) followed by a size exclusion column (Amersham), according to published procedures (38).

Apoptosis Assays. Cells were plated at a density of 150,000–200,000 cells/well in 12-well plates and, after overnight attachment, were treated with TRAIL (2–100 ng/ml) either in the absence or presence of calphostin c (an inhibitor of both classic (α, β, γ) and novel (δ, ε, η, θ) PKC isoforms; 0.1–0.25 μM; Ref. 39), or Go6976 (inhibitor of classic PKC isoforms; 1–20 μM; Ref. 40), or the PKCδ inhibitor rottlerin (1–10 μM) for up to 24 h. GC/c1 cells were pretreated for 2 h with rottlerin (2–10 μM) and subsequently cotreated with TRAIL (0.5–2 ng/ml) for 16 h. RKO cells were also treated with TRAIL (5–50 ng/ml) simultaneously and after 2 h pretreatment with the caspase inhibitors z-VAD-fmk (50 μM), z-DEVD-fmk (20 μM), or the control z-FA-fmk (20 μM; Enzyme Systems Products) for 24 h before the determination of the extent of apoptosis. Both the floating cells and the attached cells were pooled after trypsinization, were fixed in 70% ethanol, and were stored at −20°C before analysis. Apoptotic cells were detected as a sub-G1 fraction after propidium iodide staining and analysis using a Becton Dickinson FACSscan (41). Alternatively, the extent of apoptosis was determined by Annexin-V-phycocyanin (Alexis) and 17-α-aminoactinomycin-D (7-AAD; Molecular Probes) using a double staining procedure (42).

Transfection of PKCδ Constructs. A full-length PKCδ cDNA (PKCδFL), a PKCδ catalytically active fragment (PKCδCF), or a PKCδ kinase-inactive fragment [PKCδCF(K-R)] cloned into a modified pSVβgal (Clontech) containing GFP was kind gifts from Dr. Donald Kufe, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA (24). HT29 cells were plated at a density of 20 × 10⁶ cells in T-175 flasks and, after overnight attachment, were transfected with 37.5 μg of the respective cDNAs in the presence of 111 μl of fugene (Roche Diagnostics) for 48 h. Cells were subsequently sorted by FACS for expression of GFP-containing cells that also contained the respective cDNA, subsequently plated at a density of 150,000 cells/well in 12-well plates, and treated with TRAIL (2 ng/ml) for an additional 16 h. The extent of apoptosis was subsequently determined by FACS analysis of the sub-G1 compartment after propidium iodide staining.

Determination of Δψm. RKO cells were transfected with Δψm probes (Molecular Probes) and subsequently analyzed by FACS for fluorescence (FL1) intensity (43).

HT29 Isogenic Cell Lines. The retroviral expression vector pMSCV-I-GFP (expressing GFP) was a kind gift from Dr. Jill M. Lahti and Dr. Vincent J. Kidd (St. Jude Children’s Research Hospital) and has been described previously (44); and pMSCV-Bcl-2 (expressing human Bcl-2 protein) was kindly provided by Dr. John Cleveland (St. Jude Children’s Research Hospital). Retroviral supernatants were prepared as described previously (45). HT29 cells were incubated overnight in a 50% mixture of RPMI 1640 and supernatant in the presence of Polybrene (8 μg/ml; Sigma). After replacement of this medium with fresh viral supernatants and culture medium, HT29 cells were incubated at 37°C for an additional 48 h. The virus-transfected cells were sorted by expression of GFP using FACS, and stable GFP-positive cells were selected. The expression of Bcl-2 was confirmed by Western blotting.

Cellular Fractionation. RKO, HT29GFP, or HT29/Bcl-2 were plated at a density of 5 × 10⁶ cells in T-162 flasks and allowed to proliferate for 3 days before treatment and then lysis and cellular fractionation into mitochondrial, nuclear, and cytosolic fractions using the ApoAlert cellular fractionation kit (Clontech) according to the manufacturer’s directions.

Western Analysis. Western analyses were conducted as described previously (16, 46). Primary antibodies to the novel PKC isoforms PKCδ, PKCe, PKCθ, and PKCγ, Bcl-2, and Bcl-2, and Bid were from Becton Dickinson, caspase-3 from Santa Cruz Biotechnology, and caspases-8 and -9 from MBL. The secondary antibody was HRP-conjugated sheep antiperoxidase IgG (Amersham). Expression of novel PKC isoforms was also determined in RKO and HCT116 cell extracts after treatment with PMA (100 nm) for 16 h. Release of cytochrome c, HtrA2/Omi, Smac/DIABLO, or AIF from the mitochondria and expression of XIAP or c-IAP1 were determined by Western analysis. Primary antibodies were: cytochrome c (Clontech), HtrA2/Omi (a generous gift from Dr. Emad Alnemri, Thomas Jefferson University, Philadelphia, PA), Smac/ DIABLO (MBL), AIF and XIAP (Santa Cruz Biotechnology), and c-IAP1 (Alexis Biochemicals). Secondary antibodies were sheep-antiperoxidase Ig-HRP or donkey antirabbit Ig-HRP (Amersham).

Adenoviral Transduction of Bel-2. Subcloning of a Bcl-2 cDNA into the pAVS6DNA adenoviral vector (Genetic Therapy, Gaithersburg, MD) and amplification of Bel-2-Adv or the vector alone (EV-Adv) have been described previously (47). GC/c1 cells were plated as described were and transduced with the Bel-2-Adv or EV-Adv (MOI = 10) for 48 h before exposure to TRAIL (0−2 ng/ml) for 16 h. Apoptosis was determined by FACS analysis of the sub-G1 compartment, as described.

RESULTS

Calphostin c but not Go6976 Sensitizes Human Colon Carcinoma Cell Lines to TRAIL. In a panel of six human colon carcinoma cell lines, calphostin c (0.1–0.25 μM), an inhibitor of both classic and novel isoforms of PKC, sensitized VRC/c1, HCT116, HT29, RKO, and HCT8 to TRAIL-induced apoptosis (Fig. 1). GC/c1 was highly sensitive to TRAIL alone in the absence of calphostin c and, hence, could not be sensitized to TRAIL-induced apoptosis in the presence of calphostin c. In VRC/c1, HCT116, RKO, or HCT8, at TRAIL concentrations that were relatively nontoxic, apoptosis was increased to >60% in the presence of calphostin c 24 h after the initiation of treatment. Similarly in HT29, calphostin c increased TRAIL-induced apoptosis to >43%. In contrast, in HT29 and TRAIL-resistant RKO and HCT8, Go6976 (5–20 μM), an inhibitor of classic PKC isoforms, did not potentiate the cytotoxic activity of TRAIL (Fig. 2), suggesting that the inhibition of classic isoforms of PKC are not involved in attenuating the sensitivity of human colon carcinoma cell lines to TRAIL-induced apoptosis. However, because calphostin c sensitized colon carcinoma cell lines to TRAIL-induced apoptosis, we subsequently examined whether novel isoforms of PKC may be involved.

Human Colon Carcinoma Cell Lines Express PKCe and PKCe. Of the four novel PKC isoforms, colon carcinoma cell lines expressed predominantly the PKCδ and PKCe isoforms, as determined by Western analysis (Fig. 3).

Rottlerin Sensitizes Cell Lines to TRAIL-induced Apoptosis. Rottlerin, an inhibitor of the novel PKC isoform PKCδ (30–34), was subsequently evaluated for the ability to sensitize human colon carcinoma cell lines to TRAIL-induced apoptosis during 24 h coinoculation (Fig. 4). Five of the six lines (except for GC/c1) were sensitized to TRAIL (10–100 ng/ml) in the presence of rottlerin (1–10 μM), with the percentage of cells undergoing apoptosis increasing to >60% in VRC/c1, HCT116, RKO, and HCT8, and to >43% in HT29. Rott-
Lack of a Role for PKCδ in Influencing TRAIL-induced Apoptosis. To elucidate the role of PKCδ in modulating the sensitivity of human colon carcinoma cell lines to TRAIL, we transfected HCT116 with an antiapoptotic full-length PKCδ cDNA (PKCδFL), a proapoptotic PKCδ catalytically active fragment PKCδCF, or a PKCδ kinase-inactive fragment [PKCδCF(K-R); Ref. 44; Fig. 5). HCT116 transfected with the vector alone was slightly more sensitive to TRAIL than was nontransfected HCT116 cells. Of interest was that neither PKCδFL nor PKCδCF influenced the sensitivity of HCT116 to TRAIL. Subsequently, HCT116 and RKO cells were treated with PMA (100 nM) for 16 h, and the effect on expression of novel PKC isoforms was examined by Western analysis (Fig. 6C). Both PKCδ and PKCε were down-regulated in the presence of PMA. When HCT116 (Fig. 6A) and RKO (Fig. 6B) were treated with PMA for 16 h to down-regulate the expression of PKCs and were subsequently treated with TRAIL for 24 h, no effect on TRAIL-induced apoptosis was detected, and PMA alone was nontoxic. Furthermore, when these cells were treated with PMA for 16 h followed by rottlerin (10 μM) and TRAIL for 24 h, sensitization to TRAIL-induced apoptosis was still obtained.

Rottlerin and Calphostin c but not Go6976 Cause Rapid Loss in Δψm in RKO Cells. In addition to its effects on PKC, rottlerin has been reported to be capable of disrupting the Δψm (35, 36). To elucidate whether rottlerin may initiate a loss in Δψm in human colon carcinoma cell lines and to further elucidate the mechanism by which rottlerin sensitizes colon carcinoma cells to TRAIL-induced apoptosis, we treated RKO cells with rottlerin (10 μM) for 4–16 h in the absence or presence of TRAIL (50 ng/ml), and we examined the effect on Δψm by FACS analysis after cellular staining with DiOC6 (Fig. 7). Under these conditions, rottlerin dramatically reduced the Δψm from 519 to a mean fluorescence intensity of 4.3 at 4 h. Additional experiments demonstrated that this change in Δψm occurred within 15 min of rottlerin treatment (data not shown). Similarly, treatment of RKO with calphostin c (0.25 μM) for 4–16 h also induced loss in Δψm, to a mean fluorescence intensity of 331 at 4 h and 299 at 16 h (Fig. 7). In contrast, 4–16 h pretreatment with Go9776 (20 μM), which had not demonstrated any influence on TRAIL-induced apoptosis in RKO (Fig. 2), did not induce a loss in Δψm in RKO cells (Fig. 7). TRAIL alone did not influence Δψm in RKO at the times examined. When RKO cells were pretreated with rottlerin (10 μM) for 15 min and subsequently treated with TRAIL (50 ng/ml), rapid apoptosis was induced within 8 h, and the cells became highly sensitive to TRAIL (Fig. 8).

Rottlerin + TRAIL Induce Release of Proapoptotic Factors from the Mitochondria and Caspase Activation in RKO Cells. In addition to the effects of rottlerin on the induction of loss in Δψm, rottlerin (10 μM; 15 min) ± TRAIL induced cytochrome c release and the release of HtrA2/Omi from the mitochondria at 8 h (Fig. 9A). The release of Smac/DIABLO and AIF was detected earlier, at 6 h after treatment with rottlerin ± TRAIL (Fig. 9A). Only when rottlerin was combined with TRAIL was decreased expression of XIAP (but not c-IAP1) determined, 8 h after TRAIL treatment (Fig. 9A). In addition,
the activation of caspases-8, -3, and -9 and cleavage of Bid were determined only when rottlerin and TRAIL were combined (Fig. 9B), and apoptosis induced by rottlerin/ H11001 TRAIL at 18 h was inhibited by inhibitors of caspase activation (Fig. 9C). Delayed cell death (36%) induced by rottlerin treatment alone (10 μM) was observed at 48 h but not at 24 h (data not shown). This may explain the earlier release of proapoptotic factors from the mitochondria at 6–8 h (Fig. 9A) in the absence of caspase cleavage (Fig. 9B) or the induction of apoptosis (Fig. 4).

**Bcl-2 Overexpression Inhibits the Release of Proapoptotic Factors into the Cytosol in HT29 Cells.** HT29 cells were transfected with pMSCV-I-GFP (HT29GFP) or pMSCV-I-GFP-Bcl-2 (HT29Bcl-2), and cells containing GFP were selected by FACS analysis. In HT29GFP, 19–30% apoptosis was obtained after 24-h exposure to increasing concentrations of TRAIL (5–50 ng/ml; Fig. 10A). Overexpression of Bcl-2 protected cells from TRAIL-induced apoptosis. After preincubation with rottlerin (10 μM) for 15 min, TRAIL (10 ng/ml) induced apoptosis in 70% of HT29GFP cells, which was reduced to 24% in the presence of Bcl-2 overexpression (HT29Bcl-2; Fig. 10B). After cellular fractionation of HT29 or HT29/Bcl-2 cells at 6 h after treatment with rottlerin for 15 min (10 μM), TRAIL (50 ng/ml) for 6 h, or rottlerin (15-min pretreatment) and coincubation with TRAIL (6 h), cells treated with rottlerin + TRAIL but not rottlerin alone demonstrated release of HtrA2/Omi, Smac/DIABLO, AIF, and cytochrome c into the cytosol with corresponding reduced expression of both XIAP and c-IAP1 (Fig. 10C). C-IAP2 was not expressed either in RKO or HT29 cell lines.

**Rottlerin Does Not Potentiate TRAIL-induced Apoptosis in Type I Cells.** In contrast to type II cells (RKO, HT29), TRAIL-induced apoptosis occurred rapidly in type I cells (GC/c1), in which after treatment with TRAIL (50 ng/ml), 78% apoptosis was induced in 2 h (Fig. 11A). Pretreatment of GC/c1 cells with rottlerin (2–10 μM) for 2 h failed to sensitize cells to TRAIL-induced apoptosis after 16-h...
factor-(30, 50). Phosphorylation by PKC isoforms can inhibit the function of death receptor expression (18, 52). PKC also functions in formation, FADD recruitment, and caspase-8 activation (49), or inhibition of DISC phosphorylation and inactivation of Bad (25, 26), inhibition of DISC 52). Furthermore, PKC isoforms have mediated these effects by loss in ROTT Cells were incubated with rottlerin (ROTT; 10 μM) for 4–16 h in either the absence or the presence of TRAIL (2–50 ng/ml) for an additional 16 h. Apoptosis was determined by FACS analysis after propidium iodide staining. Data represent the mean ± SD of duplicate determinations. C, down-regulation of novel PKC isoforms was also determined in RKO and HCT116 cell extracts by Western analysis after treatment with PMA (100 nM) for 16 h.

Fig. 6. A, HCT116 or B, RKO cells were treated with PMA (100 nM) for 16 h before pretreatment with rottlerin (ROTT; 10 μM) for 2 h in either the absence or the presence of TRAIL (2–50 ng/ml) for an additional 16 h. Apoptosis was determined by FACS analysis after propidium iodide staining. Data represent the mean ± SD of duplicate determinations. C, down-regulation of novel PKC isoforms was also determined in RKO and HCT116 cell extracts by Western analysis after treatment with PMA (100 nM) for 16 h.

Fig. 7. Rottlerin and calphostin c but not Go6976 induce loss in Δψm in RKO cells. Cells were incubated with rottlerin (ROTT; 10 μM), calphostin c (Cal c, 0.25 μM), or Go6976 (20 μM) for 4–16 h in either the absence or the presence of TRAIL (50 ng/ml) for 4–16 h, followed by incubation with DIOC6 (2 μM) for 15 min before an analysis of loss in Δψm by FACS. FL1, fluorescence intensity.

exposure to TRAIL (0.5–2 ng/ml; Fig. 11B). Furthermore, overexpression of Bcl-2 after transduction of cells with a Bcl-2 adenovirus (MOI = 10) also did not protect cells from TRAIL-induced apoptosis (Fig. 11C).

DISCUSSION

PKC has demonstrated a role in the attenuation of apoptosis induced by ligation of death receptors of the TNFR superfamily, such that several PKC inhibitors have sensitized cells to Fas- or TRAIL-induced apoptosis. Thus, the general PKC inhibitor bisindoylmaleimide VIII sensitized to Fas-mediated apoptosis human astrocytoma 1321N1 cells and Molt4 cells that were devoid of apoptotic responses induced by anti-Fas antibody in the absence of the compound (48). Inhibition of classic isoforms of PKC by Go6976 has also enhanced anti-Fas-mediated apoptosis in Jurkat cells (49). PKC is considered to promote survival responses via the activation of nuclear factor-κB (30, 50). Phosphorylation by PKC isoforms can inhibit the function of inhibitor of nuclear factor-κB (IkBα) in the activation of nuclear factor-κB (51), and can negatively regulate TNF family receptors (18, 52). Furthermore, PKC isoforms have mediated these effects by phosphorylation and inactivation of Bad (25, 26), inhibition of DISC formation, FADD recruitment, and caspase-8 activation (49), or inhibition of death receptor expression (18, 52). PKC also functions in type II cells that require amplification via the mitochondria in induction of an apoptotic response, and not in type I cells (53).

In the present study, human colon carcinoma cell lines demonstrated a wide spectrum of sensitivity to TRAIL, demonstrating exquisite sensitivity (GC3/c1) or innate resistance (RKO, HCT8) to the ligand. The high degree of TRAIL-induced apoptosis obtained in five of the six cell lines when TRAIL was co-incubated with calphostin c, which inhibits both classic and novel isoforms of PKC, and the lack of effect of Go6976, which inhibits only the classic isoforms of PKC, suggested that novel PKCs may be important in the attenuation of TRAIL-induced apoptosis in this cell type. In this regard, a similar degree of sensitization to TRAIL-induced apoptosis in the presence of the PKC8 inhibitor, rottlerin, and the expression of PKC8 in each cell line, suggested that this isoform of PKC may be important in influencing TRAIL-induced apoptosis. However, transfection of PKC8...
inhibitor of PKCδ (35). In the RKO cells examined, rottlerin induced a dramatic loss in Δψm, which was clearly independent of its role as an inhibitor of PKCδ. Of interest was that calphostin C, which also sensitized five of six colon carcinoma cell lines to TRAIL-induced apoptosis, also induced loss of Δψm in RKO cells, in contrast to Go6976, which did not sensitize RKO to TRAIL and did not induce loss in Δψm. These data suggest that disruption of the Δψm plays a major role in TRAIL signaling and the subsequent induction of apoptosis. Of interest was the exquisite sensitivity of GC3/c1 to TRAIL, which was not further sensitized in the presence of either calphostim C or rottlerin. This is consistent with a type I signaling mechanism for TRAIL in GC3/c1, in which apoptosis commenced rapidly, at 2 h; and, in addition, overexpression of Bcl-2 did not protect cells from TRAIL-induced apoptosis, further indicating the requirement for mitochondrial involvement in rottlerin-induced sensitization to TRAIL-induced apoptosis in type II cells.

Decreased Δψm results in changes in the inner mitochondrial membrane function accompanied by an increase in outer membrane permeability, leading to the release of soluble intermembrane proteins that promote cell death into the cytosol (43), including cytochrome c (59, 60), HtrA2/Omi (61–63), Smac/DIABLO (64, 65), and AIF (66, 67). Cytochrome c forms a multimeric complex with Apaf-1 in an ATP-requiring reaction, subsequently recruiting caspase-9, followed by activation of caspase-9 and activation of downstream caspases (68, 69). Several members of the IAP family including XIAP, c-IAP1, and c-IAP2 are potent direct inhibitors of caspases-3, -7, and -9 thereby blocking enzymatic activity, XIAP being the most potent (70). Mitochondrial release of HtrA2/Omi (61–63) or Smac/DIABLO (64, 65) destabilizes these complexes, leading to the quenching of the caspase-inhibitory function of IAPs. Furthermore, the apoptosis-inducing factor AIF may play a role in caspase-independent cell death (43, 67) and activate DNases (71). In RKO cells, rottlerin treatment, either alone or in combination with TRAIL, caused the release of cyto-

Other sections of the text discuss the phosphorylation of PKC, the role of PKC in apoptosis signaling, the interaction with mitochondria, and the inhibition of tyrosine phosphorylation. The text also mentions the involvement of PKC in the regulation of apoptosis and the role of PKC inhibitors in sensitizing cells to TRAIL. The text includes references to various studies and experiments that support the conclusions drawn.

Fig. 10. HT29 isogenic cell lines HT29GFP or HT29 overexpressing Bcl-2 (HT29Bcl-2) were treated with TRAIL (5–50 ng/ml) in either the absence or the presence of rottlerin (10 µM) pretreatment for 15 min. Apoptosis was analyzed, in duplicate, at 18 h as a sub-G1 fraction by FACS. A, expression of Bcl-2 was examined by Western analysis as described in “Materials and Methods.” B, HT29 or HT29-Bcl-2 cells were treated with rottlerin (10 µM) for 15 min or TRAIL (50 ng/ml) for 6 h or were pretreated with rottlerin for 15 min before TRAIL for an additional 6 h. Cells were disrupted and separated into mitochondrial and cytosolic fractions, and the release of proapoptotic factors from the mitochondrial into the cytosol, or expression of XIAP and c-IAP1, were determined by Western analysis as described in “Materials and Methods.”

Fig. 11. A, GC3/c1 cells were treated with TRAIL (50 ng/ml) and apoptosis, were determined as a sub-G1 compartment by FACS analysis, and were determined for up to a SOG of two determinations. B, GC3/c1 cells were pretreated with rottlerin (2–10 µM) for 2 h followed by cotreatment with TRAIL (0.5–2 ng/ml) for 16 h before determination of the extent of apoptosis (mean ± SD of duplicate determinations). C, GC3/c1 cells were transduced with EV-Adv or Bcl-2-Adv (MOI = 10) for 48 h before treatment with TRAIL (0.5–2 ng/ml) for 16 h, followed by analysis of the extent of apoptosis (mean ± SD of two determinations per point). D, expression of Bcl-2, determined by Western analysis, in GC3/c1, GC3/EV (vector control), or GC3/Bcl-2 (overexpressing Bcl-2).
The induction of apoptosis in RKO cells induced by rottlerin in combination with TRAIL could be completely inhibited by the caspase inhibitors z-VAD-fmk and z-DEVD-fmk. Further evidence of apoptosis induced by rottlerin + TRAIL requiring mitochondrial involvement was demonstrated in the HT29 isogenic cell line HT29Bc2, in which overexpression of Bcl-2 inhibited TRAIL-induced apoptosis in both the absence and the presence of rottlerin, consistent with the effect of Bcl-2 in inhibiting apoptosis induced by mitochondrial uncoupling (72). In addition, the release of cytochrome c, HtrA2/Omi, Smac/DIABLO, and AIF into the cytosol, and reduced expression of the IAP protein c-IAP1 (but not XIAP) occurred only when TRAIL and rottlerin were combined, and these effects were also inhibited in the presence of Bcl-2 overexpression.

In summary, we have demonstrated that rottlerin is effective in sensitizing human colon carcinoma cell lines to TRAIL-induced apoptosis via direct effects at the level of the mitochondria, prevented by inhibitors of caspase activation, and inhibited by Bcl-2, independent of its effects as an inhibitor of the novel PKC isoform PKCδ. The finding that TRAIL resistance can be overcome by initially inducing changes in the Δψm in TRAIL-resistant cells followed by administration of TRAIL suggests that the mitochondria may constitute an important target in sensitizing human colon carcinoma cells to therapeutic approaches involving cytotoxic ligands of the TNF family. Additional studies on the relationship between loss in Δψm and events leading to the induction of apoptosis, therefore, appear warranted.

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Rottlerin Sensitizes Colon Carcinoma Cells to Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis via Uncoupling of the Mitochondria Independent of Protein Kinase C


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