

ORIGINAL PAPER

Rottlerin sensitizes glioma cells to TRAIL-induced apoptosis by inhibition of Cdc2 and the subsequent downregulation of survivin and XIAPEun Hee Kim¹, Seung U Kim² and Kyeong Sook Choi^{*1,2}¹Institute for Medical Sciences, Ajou University School of Medicine, Suwon 442-749, South Korea; ²Brain Disease Research Center, Ajou University School of Medicine, Suwon 442-749, South Korea

In the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant glioma cells, treatment with TRAIL in combination with subtoxic doses of rottlerin induced rapid apoptosis. While the proteolytic processing of procaspase-3 by TRAIL was partially blocked in these cells, treatment with rottlerin efficiently recovered TRAIL-induced activation of caspases. Treatment with rottlerin significantly decreased Cdc2 activity through the downregulation of cyclin A, cyclin B, and Cdc2 proteins, whereas the sensitizing effect of rottlerin on TRAIL-induced apoptosis was independent of PKC δ activity. Furthermore, treatment with rottlerin downregulated the protein levels of survivin and X-chromosome-linked IAP (XIAP), two major caspase inhibitors. Forced expression of Cdc2 together with cyclin B attenuated rottlerin-potentiated TRAIL-induced apoptosis by over-riding the rottlerin-mediated downregulation of survivin and XIAP protein levels. Taken together, inhibition of Cdc2 activity and the subsequent downregulation of survivin and XIAP by subtoxic doses of rottlerin contribute to amplification of caspase cascades, thereby overcoming resistance of glioma cells to TRAIL-mediated apoptosis. Since rottlerin can sensitize Bcl-2- or Bcl-xL-overexpressing glioma cells but not human astrocytes to TRAIL-induced apoptosis, this combined treatment may offer an attractive strategy for safely treating resistant gliomas.

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Keywords: TRAIL; rottlerin; apoptosis; glioma

Introduction

Malignant gliomas, which are common primary tumors of the central nervous system, are refractory to traditional chemotherapy and radiotherapeutic methods and have poor prognosis (DeAngelis, 2001). The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), initially identified by its high sequence

homology to other TNF family members (Wiley *et al.*, 1995), is considered a promising anticancer agent due to its ability to induce apoptosis in a variety of tumor cell types with only negligible effects on normal cells (Sheridan *et al.*, 1997; Ashkenazi *et al.*, 1999). However, malignant gliomas exhibit considerable heterogeneity in their sensitivity to TRAIL, even among those expressing the TRAIL-responsive cognate death receptors, DR4 and DR5 (Hao *et al.*, 2001; Knight *et al.*, 2001). Overexpression of inhibitor of apoptosis (IAP) protein family members, including survivin and X-chromosome-linked IAP (XIAP), or enhanced activity of Akt have been reported to confer resistance to TRAIL-induced apoptosis in some cancer cells (Chen *et al.*, 2001; Griffith *et al.*, 2002; Ng *et al.*, 2002). Both survivin and XIAP are overexpressed in gliomas, and their high expression is correlated with abbreviated patient survival, unfavorable prognosis, resistance to therapy, and accelerated rates of recurrences (Wagenknecht *et al.*, 1999; Chakravarti *et al.*, 2002). Many glioma cells show enhanced Akt activity induced by mutation of PTEN, which is associated with decreased susceptibility to apoptosis (Smith *et al.*, 2001; Joy *et al.*, 2003). Researchers are currently seeking to isolate the key survival signals responsible for protecting glioma cells from TRAIL-induced apoptosis and identify sensitizing agents capable of overcoming TRAIL resistance in these cells. Identification of such factors will facilitate the establishment of TRAIL-based combination regimens for the improved treatment of gliomas.

PKC comprises a family of phospholipid-dependent serine–threonine kinases that play important roles in signal transduction and in the regulation of cell growth, differentiation, and apoptosis (Dempsey *et al.*, 2000). PKC consists of 12 different isoforms, which have been classified into three major groups based on their structures and on their activation mechanisms: conventional (α , β 1, β 2, γ), novel (γ , ϵ , η , θ), and atypical (ξ , ν , λ , β) (Way *et al.*, 2000). Several PKC isoforms have been demonstrated to play roles in the regulation of death receptor-induced apoptosis (Toth *et al.*, 1999; Trauzold *et al.*, 2003). Activation of PKC by phorbol esters inhibited Fas- and TRAIL-mediated apoptosis, whereas inhibition of PKC has enhanced Fas- and TRAIL-induced cell death (Gomez-Angelats and Cidlowski, 2001; Meng *et al.*, 2002; Harper *et al.*, 2003). Although rottlerin was originally identified as a specific inhibitor

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of the novel PKC isoform, PKC δ (Gschwendt *et al.*, 1994), and was shown to inhibit specifically the activity of PKC δ in immunokinase assays (Basu *et al.*, 2001), a recent study showed that rottlerin sensitized colon carcinoma cells to TRAIL via uncoupling of the mitochondria, in a manner independent of its ability to inhibit PKC δ (Tillman *et al.*, 2003). However, the underlying mechanisms by which rottlerin sensitizes cancer cells to TRAIL-mediated apoptosis remain largely unknown. In the present study, we investigated the effect of rottlerin on TRAIL-induced apoptosis in human astrocytes and various glioma cell lines. Rottlerin could significantly sensitize TRAIL-resistant glioma cells but not human astrocytes to TRAIL-induced apoptosis. Furthermore, combined treatment with TRAIL and rottlerin was cytotoxic to glioma cells overexpressing Bcl-2 or Bcl-xL. Together, our results provide the first evidence that rottlerin can recover TRAIL sensitivity in TRAIL-resistant gliomas through inhibition of Cdc2 kinase activity and the subsequent downregulation of survivin and XIAP, in a PKC δ activity-independent manner.

Results

Subtoxic doses of rottlerin significantly enhance cell death in TRAIL-resistant human glioma cell lines but not in astrocytes

The cytotoxic activity of human recombinant soluble TRAIL was tested in four glioma cell lines: U87MG, A172, T98G, and U251MG (Figure 1a). Treatment with 50–200 ng/ml TRAIL induced a limited cell death (<15%) over 48 h, suggesting that these cells are resistant to the apoptotic effects of TRAIL. Next, we tested the cytotoxic effect of rottlerin alone or in combination with TRAIL. Rottlerin alone did not induce any morphological signs of cell death up to 10 μ M. However, cell viability was significantly reduced by the combined treatment both when holding the concentration of TRAIL fixed and varying the concentrations of rottlerin and, conversely, when holding the concentration or rottlerin fixed and varying TRAIL (Figure 1b). Next, we investigated whether the combined treatment with rottlerin and TRAIL affects the viability of normal astrocytes. The astrocytes were resistant to 200 ng/ml TRAIL and their viability was not significantly affected by the combined treatment with varied concentrations of rottlerin and TRAIL for 24 h (Figure 1c). These results suggest that sensitizing regimens using rottlerin with TRAIL may be preferentially toxic for glioma cells over normal astrocytes.

Critical role of caspases in cell death by the combined treatment with rottlerin and TRAIL

We examined whether the rottlerin-facilitated TRAIL-induced cell death of glioma cells was mediated through caspase(s). U87MG cells were treated with 10 μ M rottlerin for 16 h, or 100 ng/ml TRAIL alone, or

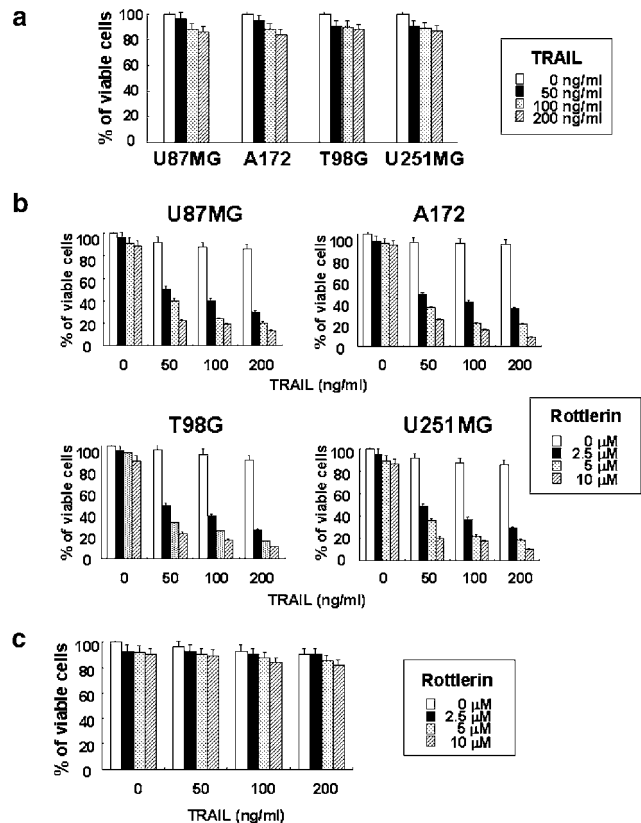


Figure 1 Subtoxic doses of rottlerin significantly sensitize TRAIL-resistant glioma cell lines to TRAIL-induced cell death. (a) Effect of TRAIL on the viability of glioma cell lines. Four glioma cell lines were treated with TRAIL at the indicated concentrations for 48 h and cellular viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values from each treatment group are expressed as a percentage relative to the untreated control (100%). (b) Effect of rottlerin and/or TRAIL on the viability of glioma cell lines. Glioma cells were treated with rottlerin for 30 min and further treated with TRAIL for 24 h at the indicated concentrations. Cellular viability was assessed using calcein-AM and Etd-1. Graphs represent the average and standard deviation of three individual experiments. (c) Human astrocytes are resistant to the effect of rottlerin and TRAIL in combination. Human primary astrocytes were treated with TRAIL in the absence or presence of rottlerin at the indicated concentrations for 24 h. Cellular viability was assessed using calcein-AM and Etd-1

pretreated with rottlerin for 30 min, followed by TRAIL for the indicated times. Treatment with 10 μ M rottlerin alone for 16 h did not induce any proteolytic processing of caspases. In response to TRAIL, the 32 kDa procaspase-3 was partially cleaved to a 20 kDa intermediate form after 4 h, but further cleavage into the active p17 subunit was neither detected nor were other caspase-processing events. However, treatment with rottlerin plus TRAIL induced the cleavage of caspase-3 into the p20 intermediate form at 4 h, and its subsequent cleavage into the active p17 subunit after 8 h. Caspase-2, -7, -8, and -9 were also progressively processed into their respective active forms after 8 h of combined treatment (Figure 2a). We further assessed the cleavage of several key death substrates that indicate activation of caspases, including FAK (caspase-3),

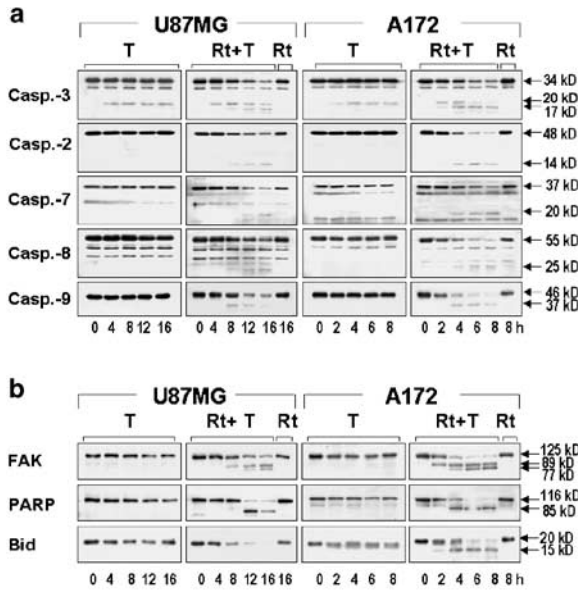


Figure 2 Activation of caspases during apoptosis induced by the combined treatment with rottlerin and TRAIL. (a) Proteolytic processing of caspases. U87MG or A172 cells were treated with 10 μ M rottlerin alone, 100 ng/ml TRAIL alone, or a combination of both for the indicated time points. Cell extracts were prepared for Western blotting to detect the changes in the expression of caspases. (b) Degradation of the substrate proteins of caspases. To confirm the activation of caspases, Western blotting of the substrate proteins was performed

PARP (substrate for caspase-3, -7), and Bid (caspase-8) (Earnshaw *et al.*, 1999). In parallel with the proteolytic processing of caspases, these substrate proteins were progressively degraded from 8 to 12 h after the combined treatment, whereas they were not degraded following treatment with TRAIL or rottlerin alone (Figure 2b). Similar but faster activation patterns of caspases were observed in A172 cells following treatment with rottlerin plus TRAIL (Figure 2a and b), consistent with the faster apoptotic progression in these cell lines compared with that in U87MG cells (Figure 1b). To determine the role of caspase(s) in the potentiation of TRAIL-induced apoptosis by rottlerin, we tested the effects of caspase inhibitors. Pretreatment of U87MG cells with z-VAD, a pancaspase inhibitor, significantly blocked cell death induced by rottlerin and TRAIL (Figure 3a). Treatment with z-IETD (which effectively inhibits caspase-8 activity) or z-DEVD (which effectively inhibits caspase-3 activity) blocked the combined treatment-induced apoptosis more potently than did z-LEHD (which effectively inhibits caspase-9 activity). Since several caspase inhibitors, including z-DEVD and z-VAD, have been recently shown to block efficiently the activity of another cysteine protease, cathepsin B (Foghsgaard *et al.*, 2001; Rozman-Pungercar *et al.*, 2003), we investigated the effect of CA-074-Me, a cathepsin B-specific inhibitor, on rottlerin-sensitized TRAIL-induced apoptosis. However, treatment with CA-074-Me did not affect apoptosis induced by the combined treatment. More-

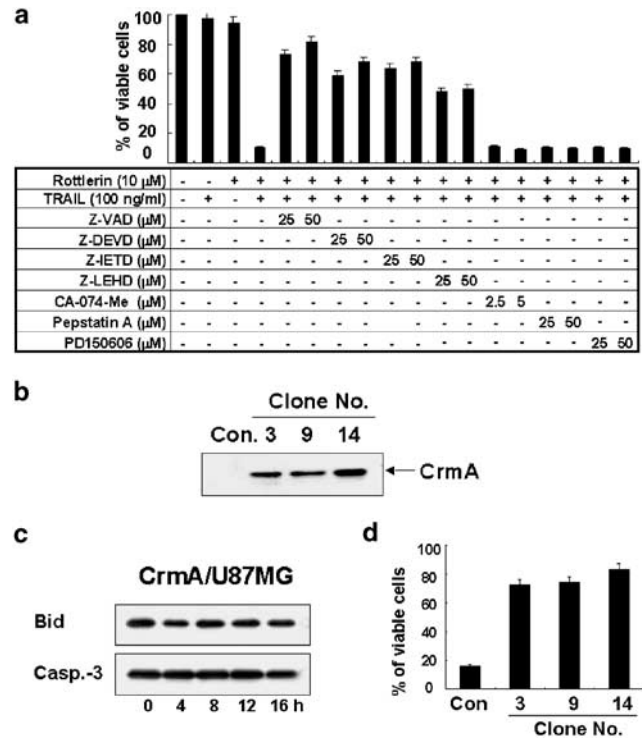


Figure 3 Caspases play a critical role in apoptosis induced by cotreatment with rottlerin and TRAIL. (a) Effect of the inhibition of the specific proteases on rottlerin-facilitated TRAIL-induced apoptosis. U87MG cells were treated with tetrapeptide inhibitors specific for caspase(s), CA-074-Me, pepstatin A, or PD 150606 at the indicated concentrations for 30 min and then further treated with 10 μ M rottlerin and 100 ng/ml TRAIL. Cellular viability was assessed using calcein-AM and Etd-1. The graph represents one of two independent experiments with consistent results. (b) Immunoblot of CrmA expression in U87MG sublines stably transfected with control vector pcDNA3 alone or with the CrmA cDNA. (c) Effect of CrmA overexpression on Bid and procaspase-3 protein levels following treatment with rottlerin and TRAIL. The sublines overexpressing CrmA (Clone no. 14) were treated with 10 μ M rottlerin and 100 ng/ml TRAIL in combination and cell extracts were prepared for Western blotting. (d) Overexpression of CrmA blocks rottlerin-facilitated TRAIL-induced apoptosis. The sublines overexpressing CrmA were treated with 10 μ M rottlerin plus 100 ng/ml TRAIL for 24 h and cellular viability was measured using calcein-AM and Etd-1. Data represent means of triplicate determinations

over, treatment with pepstatin A (a cathepsin D-specific inhibitor) or PD 150606 (a calpain-specific inhibitor) was also unable to block this apoptosis. Similar results were obtained in the experiments using A172 cells (data not shown). Next, we found that overexpression of CrmA, viral caspase-8 inhibitor (Zhou *et al.*, 1997), inhibited the proteolytic processing of both Bid, the substrate protein of caspase-8, and procaspase-3 by the combined treatment (Figure 3b and c). Furthermore, rottlerin-facilitated TRAIL-induced apoptosis was significantly blocked in these cells overexpressing CrmA (Figure 3d). Taken together, these results suggest that caspases play the critical role in apoptosis by combined treatment with rottlerin and TRAIL.

Rottlerin sensitizes glioma cells to TRAIL-induced apoptosis independent of the activities of PKC δ and stress-activated MAP kinases

Since rottlerin was originally identified as a specific inhibitor of the novel PKC isoform, PKC δ (Gschwendt *et al.*, 1994), we attempted to clarify whether the effect of rottlerin on TRAIL-induced apoptosis was directly associated with inhibition of PKC δ activity. First, we tested whether overexpression of PKC δ could over-ride the sensitizing effect of rottlerin on TRAIL-induced apoptosis. U87MG cells were transiently transfected with plasmids encoding wild-type (WT) PKC δ or control pcDNA3, and then treated with rottlerin plus TRAIL (Figure 4a). While overexpression of WT PKC δ in U87MG cells increased PKC δ kinase activity on MBP, it did not alleviate rottlerin-induced sensitivity to TRAIL-induced apoptosis. Next, we examined whether overexpression of a dominant-negative (DN) mutant of PKC δ could mimic the sensitizing effect of rottlerin on TRAIL-induced apoptosis. Treatment of U87MG cells with TRAIL alone after transient transfection with plasmids encoding the DN PKC δ mutant did not induce any significant cell death. We further examined whether treatment with PMA affected TRAIL-induced apoptosis or rottlerin-stimulated TRAIL-induced apoptosis. When U87MG or A172 cells were treated with PMA for 16 h followed by treatment with TRAIL for 24 h, no effect on TRAIL-induced apoptosis was detected. Furthermore, when these cells were treated with PMA for 16 h followed by rottlerin plus TRAIL for 24 h, sensitization to TRAIL-induced apoptosis was still obtained (Figure 4b). Pretreatment with PMA for 30 min also demonstrated a similar effect on TRAIL-mediated apoptosis and apoptosis induced by rottlerin plus TRAIL (data not shown). Since the attempts to modulate PKC δ activity by PMA treatment or forced expression of WT or DN PKC δ did not affect TRAIL-mediated apoptosis or rottlerin-stimulated TRAIL-induced apoptosis, we further examined the endogenous protein levels of PKC δ in glioma cells (Figure 4c). Compared with HeLa cervical carcinoma cells and HCT116 colon cancer cells, glioma cells contained very low levels of PKC δ protein. Taken together, our results demonstrate that rottlerin sensitizes glioma cells to TRAIL-induced apoptosis independent of its effect as a PKC δ inhibitor.

Recently, Ohtsuka and Zhou (2002) reported that bisindolylmaleimide VII (a PKC inhibitor) enhanced DR5-mediated apoptosis through the JNK/p38 kinase pathway. Therefore, we investigated the involvement of these stress-activated protein kinases in rottlerin-facilitated TRAIL-induced apoptosis. Changes in the activities of p38 and JNK after treatment with rottlerin and/or TRAIL were examined by Western blot analysis; their active forms were detected with the respective phospho-specific antibodies (Figure 5). Neither p38 nor JNK was activated in response to TRAIL and/or rottlerin, suggesting that p38 and JNK may not be involved in rottlerin-potentiated TRAIL-induced apoptosis.

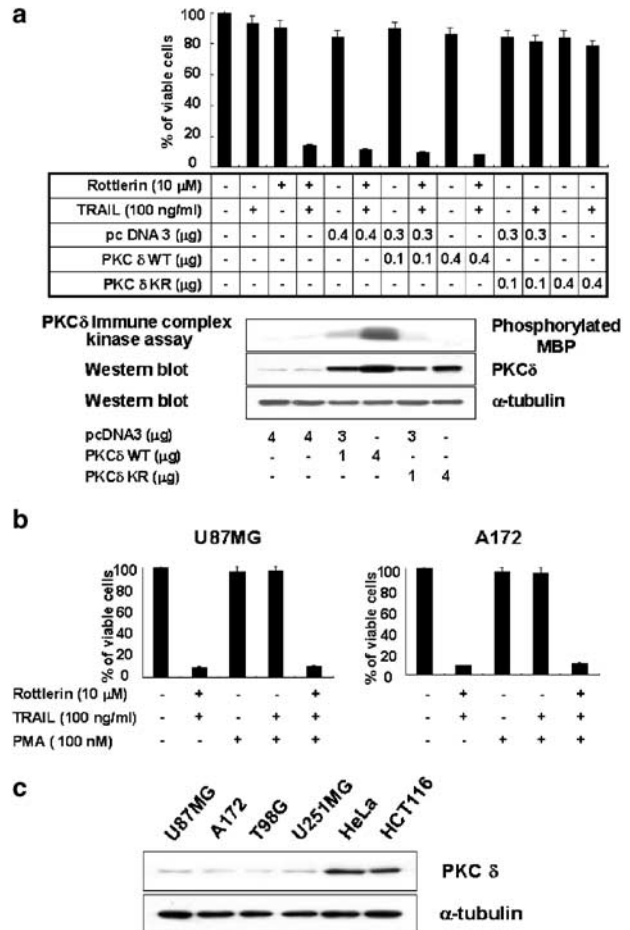


Figure 4 Rottlerin sensitizes glioma cells to TRAIL-induced apoptosis independent of PKC δ activity. (a) Effect of WT PKC δ expression on rottlerin-stimulated TRAIL-induced apoptosis and effect of DN PKC δ expression on TRAIL-induced apoptosis. To examine whether forced expression of WT PKC δ could over-ride rottlerin-potentiated TRAIL-induced apoptosis, U87MG cells (seeded in 24-well plates) were transiently transfected with plasmids encoding WT PKC δ or pcDNA3 at the indicated concentrations. After 24 h, cells were treated with rottlerin and TRAIL for another 24 h or left untreated. Cellular viability was assessed using calcein-AM and Etd-1. To examine whether expression of DN PKC δ could mimic the sensitizing effect of rottlerin, U87MG cells were transiently transfected with plasmids encoding DN PKC δ (PKC δ KR) or pcDNA3. After 24 h, cells were treated with TRAIL for another 24 h or left untreated. Cellular viability was assessed using calcein-AM and Etd-1. The data represent one of three independent experiments with consistent results. To confirm whether transfected WT PKC δ or DN PKC δ was actually expressed and active in U87MG cells, PKC δ activities in cells transfected with pcDNA3, WT or DN PKC δ were analysed by immune complex kinase assay on MBP as described in Materials and methods. Total protein levels of PKC δ in transfected cells were analysed by Western blotting using an anti-PKC δ antibody. (b) Effect of PMA treatment on TRAIL-induced apoptosis or rottlerin-stimulated TRAIL-induced apoptosis. U87MG or A172 cells were pretreated with 100 nM PMA for 16 h before treatment with 100 ng/ml TRAIL alone or 10 μ M rottlerin plus 100 ng/ml TRAIL for 24 h. Cellular viability was assessed using calcein-AM and Etd-1. Data represent the mean \pm s.d. of duplicate determinations. (c) Western blot analysis to detect the endogenous protein levels of PKC δ in glioma cells, HeLa, and HCT116 cells. Equal protein amounts (40 μ g) from the respective cells were separated by 10% SDS-PAGE, and Western blotting was performed using anti-PKC δ or α -tubulin antibody

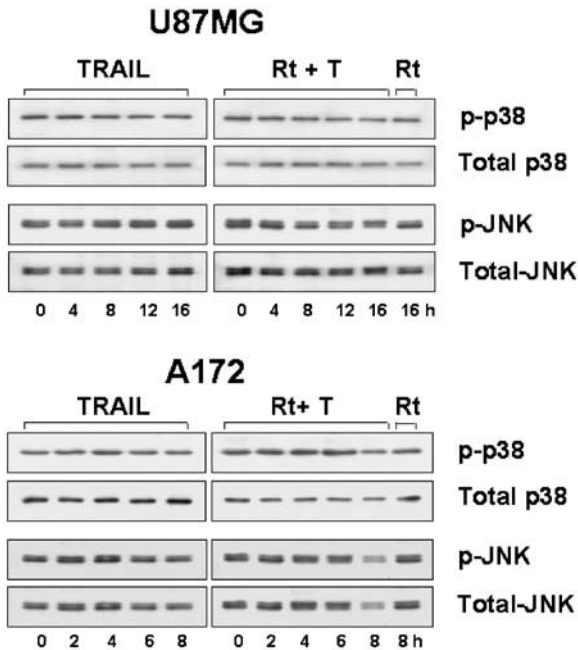


Figure 5 Activation of p38 or JNK is not involved in rottlerin-stimulated TRAIL-induced apoptosis. U87MG or A172 cells were treated with 10 μ M rottlerin alone, 100 ng/ml TRAIL alone, or a combination of both for the indicated time points. Cell extracts were prepared for Western blotting to detect the changes in p38 and JNK activities and their protein levels

Overexpression of Bcl-2 or Bcl-xL does not block rottlerin-potentiated TRAIL-induced apoptosis in glioma cells

Recently, Tillman *et al.* (2003) have reported that rottlerin affects mitochondrial function, thereby sensitizing colon cancer cells to TRAIL and proposed that mitochondria are an important target for overcoming inherent resistance to TRAIL. Therefore, we examined whether overexpression of Bcl-2 or Bcl-xL, which had previously been shown to prevent mitochondrial alterations in response to various death stimuli (Adams and Corry, 1998), could block apoptosis induced by rottlerin and TRAIL cotreatment in glioma cells. The cellular viability of Bcl-2- or Bcl-xL-overexpressing U87MG sublines cotreated for 24 h was assessed with calcein-AM and ethidium homodimer-1 (Etd-1) (Figure 6a and b). TRAIL-induced apoptosis in the presence of rottlerin was not interrupted by overexpression of either Bcl-2 or Bcl-xL, indicating that the mitochondrial apoptotic pathways operating via caspase-9 may not play a critical role in rottlerin-sensitized TRAIL-induced apoptosis in the tested glioma cells.

Combined treatment with rottlerin and TRAIL reduces expression of multiple proteins associated with cell survival

To explore the underlying mechanisms by which rottlerin sensitizes TRAIL-induced apoptosis in glioma cells, we analysed the expression of several apoptosis-

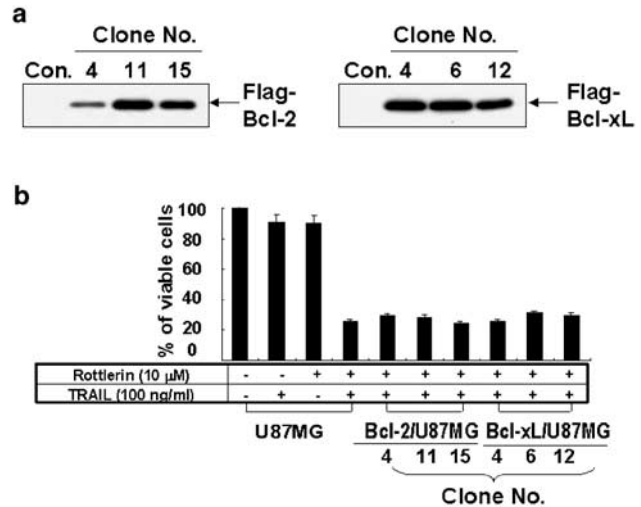


Figure 6 Effect of rottlerin and TRAIL cotreatment on the cellular viability of Bcl-2- and Bcl-xL-overexpressing cells. (a) Analysis of Bcl-2 or Bcl-xL expression in the stably transfected cell lines. Western blotting using an anti-Flag antibody was performed to confirm the overexpression of Flag-tagged Bcl-2 or Flag-tagged Bcl-xL in the selected cell lines. (b) Forced Bcl-2 or Bcl-xL expression does not attenuate rottlerin-facilitated TRAIL-induced cell death. U87MG cells overexpressing Bcl-2 or Bcl-xL were treated with 10 μ M rottlerin plus 100 ng/ml TRAIL for 24 h and cellular viability was measured by calcein-AM and Etd-1

related proteins following treatment with rottlerin and TRAIL. The combined treatment did neither change the expression levels of Bcl-2, Bcl-xL, c-IAP2, and FLICE-inhibitory protein (FLIP) in these cells nor were the protein levels of DR4 and DR5 altered by the combined treatment. In contrast, the protein levels of survivin, XIAP, and the activated form of Akt were significantly decreased in both U87MG and A172 cells treated with rottlerin and TRAIL (Figure 7a). Both XIAP and Akt are reportedly cleaved by caspases during apoptosis (Deveraux *et al.*, 1999a; Rokudai *et al.*, 2000). Therefore, we investigated whether the observed decrease in these protein levels during rottlerin/TRAIL-induced apoptosis might result from enhanced caspase activity. Interestingly, overexpression of CrmA did not block downregulation of survivin, XIAP, or phospho-Akt, although a slight observed decrease in total Akt protein levels induced by the combined treatment was inhibited by CrmA overexpression (Figure 7b). These results suggest that rottlerin may downregulate these proteins via unknown caspase-independent regulatory mechanisms.

Rottlerin enhances TRAIL-induced apoptosis by downregulation of survivin and XIAP

We further examined whether rottlerin itself downregulates survivin, XIAP protein levels, and/or Akt activity. Treatment with rottlerin alone led to a significant dose- and time-dependent decrease in survivin protein levels, and to a lesser extent, although still

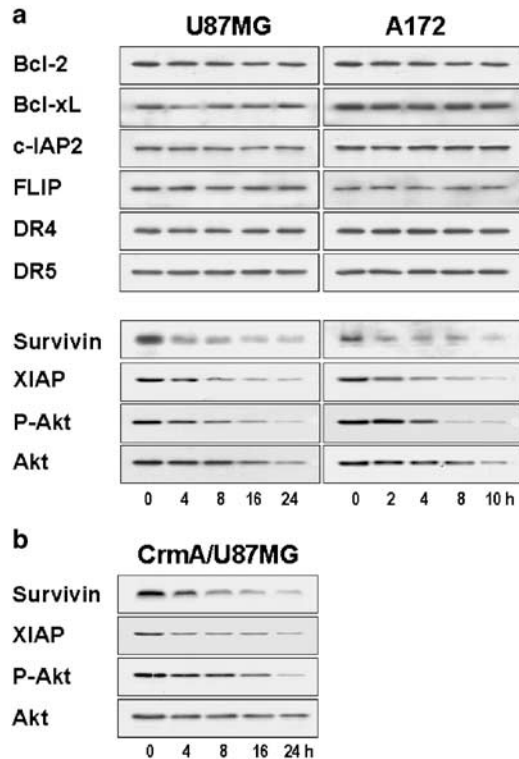


Figure 7 Effect of the combined treatment with rottlerin and TRAIL on the expression of multiple proteins associated with cell survival. **(a)** Changes in the expression of the intracellular regulators of apoptosis and proteins associated with TRAIL signaling pathway. Cell extracts were prepared from U87MG or A172 cells treated with $10 \mu\text{M}$ rottlerin and 100 ng/ml TRAIL for the indicated times and Western blotting was performed. **(b)** Effect of CrmA overexpression on survivin, XIAP, and Akt protein levels following treatment with rottlerin and TRAIL. Sublines overexpressing CrmA (Clone no.14) were treated with $10 \mu\text{M}$ rottlerin and 100 ng/ml TRAIL in combination and cell extracts were prepared for Western blotting

dose- and time-dependent decrease in XIAP protein levels. The activated form of Akt also significantly decreased in a dose- and time-dependent manner, although the total protein levels of Akt were not altered (Figure 8a). To examine whether this downregulation of survivin, XIAP protein, and/or Akt activity by rottlerin is critical to trigger TRAIL-induced apoptosis, we established stable cell lines overexpressing survivin, XIAP, or activated Akt (Figure 8b). Overexpression of survivin or XIAP attenuated rottlerin-facilitated TRAIL-induced apoptosis, while overexpression of active Akt did not (Figure 8c). These results suggest that downregulation of survivin or XIAP by rottlerin, but not Akt, may be involved in overcoming TRAIL-resistance in glioma cells. To further investigate whether the difference in survivin and/or XIAP expression triggered by rottlerin treatment accounted for its effect on TRAIL sensitivity (not only in control cells but also in cells overexpressing survivin or XIAP), we analysed the changes in the total protein levels of survivin and XIAP (Figure 8d). No significant change was observed

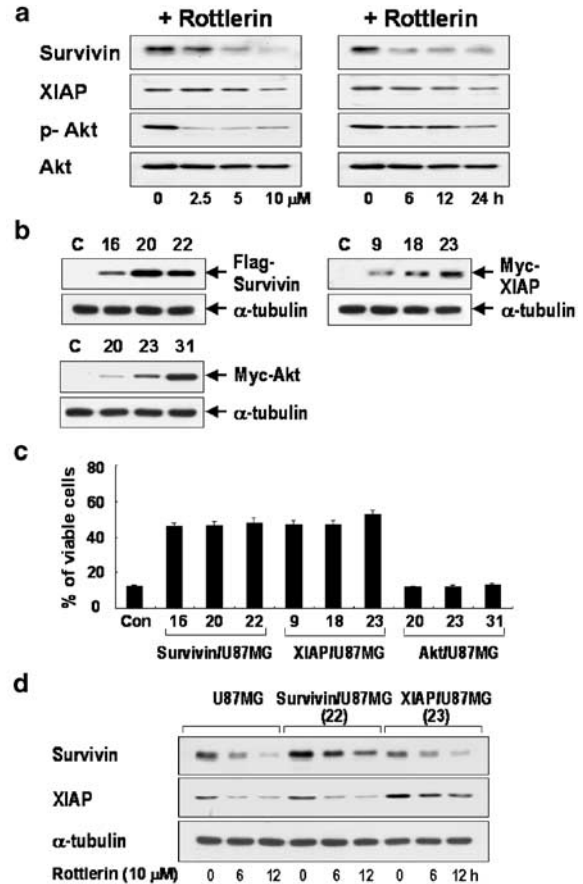


Figure 8 Rottlerin enhances TRAIL-induced apoptosis by downregulation of survivin and XIAP. **(a)** Effect of rottlerin on survivin, XIAP, and Akt protein levels. U87MG cells were treated with rottlerin as indicated and cell extracts were prepared for Western blotting of the indicated proteins. **(b)** Analysis of survivin, XIAP or Akt expression in the stably transfected cell lines. Western blotting using an anti-Flag antibody or anti-Myc antibody was performed to detect the protein levels of Flag-tagged survivin, Myc-tagged XIAP, or Myc-tagged active Akt in the selected cell lines. **(c)** Effect of survivin, XIAP, or active Akt overexpression on rottlerin-facilitated TRAIL-induced cell death. Control U87MG cells and U87MG sublines overexpressing survivin, sublines overexpressing XIAP, or U87MG sublines overexpressing active Akt were treated with $10 \mu\text{M}$ rottlerin plus 100 ng/ml TRAIL for 24 h and cellular viability was measured using calcein-AM and Etd-1. Values from each treatment group are expressed as a percentage relative to the untreated control U87MG cells (100%). Data represent means of triplicate determinations. **(d)** Effect of rottlerin on the protein levels of survivin and XIAP. U87MG, sublines overexpressing survivin (Clone no. 22) and sublines overexpressing XIAP (Clone no. 23) were treated with $10 \mu\text{M}$ rottlerin for the indicated time points. Western blotting was performed to detect the protein levels of survivin, XIAP, and α -tubulin

in rottlerin-mediated downregulation of survivin in the stable cell lines overexpressing XIAP, nor was any change observed in the downregulation of XIAP in the stable cell lines overexpressing survivin. However, the total protein levels of survivin in the rottlerin-treated stable cell lines overexpressing survivin still remained as high as those in untreated parental cells, despite the rottlerin-mediated downregulation of endogenous survivin. Moreover, the stable cell lines overexpressing

XIAP also retained XIAP protein levels comparable to those seen in untreated cells, despite the rottlerin-mediated downregulation. Taken together, these results further support our hypothesis that downregulation of survivin and XIAP causes sensitization to TRAIL.

Rottlerin inhibits Cdc2 kinase activity through downregulation of cyclin A, cyclin B, and Cdc2 protein levels

Next, we investigated the upstream signaling pathways leading to rottlerin-induced downregulation of survivin and XIAP. Since we recently showed that inhibition of Cdc2 kinase activity sensitizes glioma cells to TRAIL-induced apoptosis by downregulation of survivin and XIAP (Kim *et al.*, 2004), we examined whether the sensitizing effect of rottlerin on TRAIL-induced apoptosis was also associated with inhibition of Cdc2 kinase. U87MG cells were treated with 10 μ M rottlerin alone, 100 ng/ml TRAIL alone, or 10 μ M rottlerin plus 100 ng/ml TRAIL for 24 h. Cdc2 kinase activity was measured by immunoprecipitation with an anti-Cdc2 antibody, followed by a kinase assay on histone H1 as an exogenous conventional substrate (Figure 9a). While Cdc2-associated histone H1 kinase activity was not affected by treatment with TRAIL alone, this activity was significantly decreased following treatment with rottlerin alone or rottlerin plus TRAIL. Next, we examined whether the rottlerin-induced reduction of Cdc2 kinase activity might result from rottlerin-induced changes in the expression of cell cycle regulators (Figure 9b). Rottlerin alone significantly downregulated the protein levels of cyclin A, cyclin B, and Cdc2 in U87MG and A172 cells in a dose- and time-dependent manner. Furthermore, pRb was hypophosphorylated by rottlerin treatment in a dose- and time-dependent manner. These results suggest that rottlerin-induced downregulation of cell cycle regulators controlling Cdc2 activity may be directly associated with the modulation of survivin and XIAP protein levels.

Forced expression of Cdc2 together with cyclin B attenuates rottlerin-potentiated TRAIL-induced apoptosis by over-riding rottlerin-mediated downregulation of survivin and XIAP protein levels

Next, we examined whether forced enhancement of Cdc2 kinase activity could recover rottlerin-mediated downregulation of survivin and XIAP protein levels. U87MG cells were transfected with expression vectors encoding cyclin A, cyclin B, Cdc2 alone, or in combination, and then treated with or left untreated. Rottlerin-induced downregulation of survivin protein levels was alleviated by overexpression of cyclin B more so than by overexpression of cyclin A or Cdc2 alone (Figure 10a). Coexpression of Cdc2 with cyclin B induced the most striking effect on recovery of survivin protein levels following treatment with rottlerin, suggesting that cyclin B-dependent Cdc2 kinase activity may be important for regulating survivin protein levels in these cells. Rottlerin-mediated downregulation of

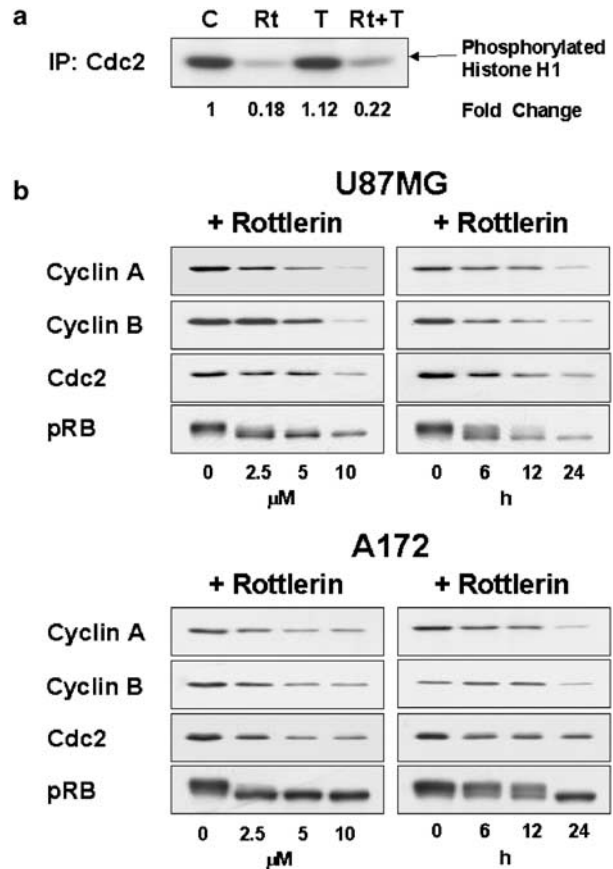


Figure 9 Inhibition of Cdc2 kinase by rottlerin. (a) Inhibition of Cdc2 kinase activity by subtoxic dose of rottlerin. U87MG cells were treated with 10 μ M rottlerin alone, 100 ng/ml TRAIL alone, or 10 μ M rottlerin plus 100 ng/ml TRAIL for 24 h. Cell extracts were prepared and Cdc2 immune complex kinase assays were performed on histone H1. (b) Changes in the expression levels of intracellular regulators of cell cycle. U87MG or A172 cells were treated with rottlerin at the indicated concentrations for 24 h or at 10 μ M for the indicated time periods and cell extracts were prepared for Western blotting of the indicated proteins

XIAP protein levels was similarly modulated by the expression of these cell cycle regulators, but to a lesser extent. We further investigated whether forced enhancement of Cdc2 kinase activity could over-ride rottlerin-potentiated TRAIL-induced apoptosis. Apoptosis induced by combined treatment with rottlerin and TRAIL was most significantly attenuated by coexpression of Cdc2 with cyclin B, while expression of Cdc2 alone, coexpression of Cdc2 and cyclin A, or coexpression of cyclin A and cyclin B, had only slight attenuating effects on rottlerin-stimulated TRAIL-induced apoptosis (Figure 10b). Taken together, our results demonstrate that treatment with rottlerin reduces Cdc2 kinase activity by suppression of the associated cell cycle regulators. Subsequent downregulation of survivin XIAP, two major IAP proteins, by reduction of Cdc2 activity may provide one mechanism by which rottlerin overcomes barriers blocking TRAIL-induced apoptosis of glioma cells.

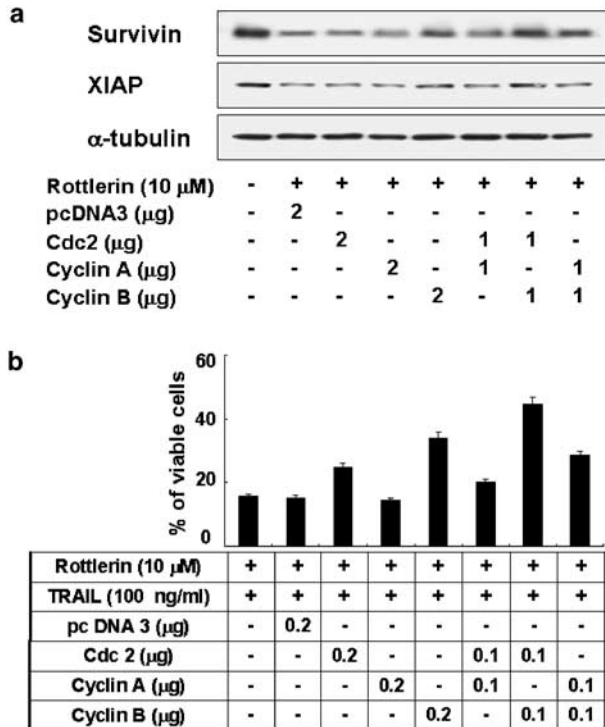


Figure 10 Forced activation of Cdc2 over-rides rottlerin-potenti-ated TRAIL-induced apoptosis by inhibition of rottlerin-mediated downregulation of survivin and XIAP. (a) Coexpression of Cdc2 and cyclin B attenuated rottlerin-mediated downregulation of survivin and XIAP. U87MG cells (5×10^5 cells/60 mm dish) were transiently transfected with plasmids encoding Cdc2, cyclin A, and cyclin B alone, or cotransfections were performed with plasmids encoding Cdc2 and cyclin A, Cdc2 and cyclin B, or cyclin A and B; transfected cells were then treated with rottlerin for 16 h. Cell extracts were prepared for Western blotting to detect survivin or XIAP. (b) Coexpression of Cdc2 and cyclin B attenuated rottlerin-mediated downregulation of survivin and XIAP. U87MG cells (5×10^4 cells/well seeded at 24-well plate) were transiently transfected with plasmids encoding Cdc2, cyclin A, and cyclin B alone or cotransfected with plasmids encoding Cdc2 and cyclin A, Cdc2 and cyclin B, or cyclin A and B; transfected cells were then treated with rottlerin for 24 h. Cellular viability was measured using calcein-AM and Etd-1. The values from each treatment group are expressed as a percentage relative to the untreated control U87MG cells (set at 100%)

Discussion

Malignant gliomas of astrocytic origin are the most common and aggressive type of human brain tumors (DeAngelis, 2001). The efficiency of TRAIL-induced apoptosis in malignant gliomas is variable, and many glioma cell lines are partially or completely resistant to this ligand (Hao *et al.*, 2001; Knight *et al.*, 2001). In our study, treatment with 100 ng/ml recombinant TRAIL (the nontagged 19 kDa protein containing the C-terminal 169 amino acids (aa 114–281) KOMA Biotech) transiently (up to 8 h) induced apoptosis in 10–15% of the glioma cells. Contrary to our results, several other groups had previously reported that these glioma cells

are sensitive to TRAIL (Rieger *et al.*, 1998; Rohn *et al.*, 2001). This discrepancy in the sensitivities of glioma cells to TRAIL might be caused by the use of different versions of recombinant TRAIL; the previous works used polyhistidine-tagged TRAIL, whereas we used nontagged TRAIL. The polyhistidine-tagged recombinant form of TRAIL has been shown to be more cytotoxic to normal hepatocytes than nontagged, soluble native-sequence TRAIL (Lawrence *et al.*, 2001). His-tagged recombinant TRAIL has been reported to be more active than nontagged recombinant TRAIL against U87MG cells *in vitro* (Fulda *et al.*, 2002). Moreover, *in vivo* treatment with nontagged TRAIL alone only temporarily delayed tumor growth (Fulda *et al.*, 2002), whereas treatment with His-tagged TRAIL alone eradicated glioma xenografts (Roth *et al.*, 1999). Taken together, these results suggest that polyhistidine-tagged recombinant soluble form of TRAIL may be more cytotoxic to both normal cells and glioma cells.

Here, we showed that many TRAIL-resistant glioma cells could be converted to TRAIL-sensitive glioma cells by cotreatment with rottlerin, which was originally identified as a specific inhibitor of PKC δ (Gschwendt *et al.*, 1994). Recent observations have implicated the PKC pathway in the protection of cells from apoptosis induced by ligation of TNFR superfamily death receptors. Activation of PKC abrogates CD95-induced apoptosis through inhibition of FADD recruitment, caspase-8 activation and subsequent DISC (death-inducing signaling complex) formation (Gomez-Angelats and Cidrowski, 2001). In addition, PKC activation by treatment with PMA inhibits apoptosis mediated by TRAIL or TNF- α in Jurkat cells by disrupting recruitment of FADD (Meng *et al.*, 2002). However, in this study, the following evidences suggest that rottlerin sensitizes glioma cells to TRAIL-induced apoptosis through a PKC δ -independent mechanism: (a) exogenously expressed WT PKC δ did not attenuate rottlerin-stimulated TRAIL-induced apoptosis (Figure 4a); (b) exogenously expressed DN PKC δ did not enhance TRAIL-induced apoptosis (Figure 4a); (c) neither TRAIL-mediated apoptosis nor rottlerin-stimulated TRAIL-induced apoptosis was affected by treatment with PMA (Figure 4b); and (d) the glioma cells used in our study expressed very low levels of PKC δ (Figure 4c). Consistent with our results, Mandil *et al.* (2001) recently reported that various malignant gliomas expressed very low or undetectable levels of PKC δ , suggesting that rottlerin-induced inhibition of PKC δ may be insignificant in glioma cells.

There is considerable controversy regarding the mechanism of action and specificity of rottlerin. It was recently reported that rottlerin acts as a mitochondrial uncoupler independent of its PKC δ inhibiting activity (Soltoff, 2001). Moreover, Tillman *et al.* (2003) recently reported that rottlerin sensitizes colon carcinoma cells to TRAIL-induced apoptosis via a PKC δ -independent uncoupling of the mitochondria. In addition, other groups have reported a variety of PKC δ -independent

actions of rottlerin. For example, rottlerin attenuated palmitate-induced apoptosis in an insulin-secreting cell line (BRIN-BD11) through a PKC δ -independent mechanism (Welters *et al.*, 2004). Moreover, rottlerin inhibited tonic-dependent expression of TonE binding protein (TonEBP) at the mRNA level (Zhao *et al.*, 2002), but inhibited astrocytic glutamate transport activity through acceleration of GLAST (glutamate aspartate transporter) protein degradation (Susarla and Robinson, 2003), indicating that rottlerin may play PKC δ -independent roles in modulating gene/protein expression. Here, we showed that rottlerin significantly downregulated cyclin A, cyclin B, and Cdc2 (Figure 9b). However, it remains to be clarified whether rottlerin targets a common transcription factor, possibly NF-Y or E2F (Zwicker *et al.*, 1995; Lavia and Jansen-Durr, 1999; Manni *et al.*, 2001), or perhaps the proteolytic system, for the downregulation of cyclin A, cyclin B, and Cdc2.

In the present study, we explored the possible underlying mechanisms by which rottlerin might sensitize glioma cells to TRAIL-induced apoptosis. The gene PTEN, which encodes a phosphoinositide phosphatase, is frequently mutated or deleted at chromosome 10q23 in malignant gliomas (Li *et al.*, 1997; Steck *et al.*, 1997), and PTEN mutations are associated with decreased survival among patients with malignant gliomas (Smith *et al.*, 2001). PTEN-deficient glioma cells often resist chemotherapy through constitutive activation of the Akt-mediated cell growth and survival pathway (Shingu *et al.*, 2003), suggesting that the PI3-K/Akt pathway may be a candidate target for anticancer therapy in glioma cells. In our study, we observed that while rottlerin downregulated Akt activity in U87MG and A172 glioma cells, overexpression of active Akt did not attenuate the cell death induced by cotreatment with rottlerin and TRAIL. Therefore, downregulation of Akt activity by rottlerin may not play a critical role in the enhancement of TRAIL-induced apoptosis. Recent reports have demonstrated that there are intracellular proteins capable of inhibiting death receptor-mediated apoptosis when present at sufficient levels (Adams and Corry, 1998; Tschopp *et al.*, 1998; Deveraux and Reed, 1999b; Thakkar *et al.*, 2001; Griffith *et al.*, 2002; Ng *et al.*, 2002). Since IAPs such as survivin, XIAP, c-IAP1, and c-IAP2 block apoptosis at the effector phase, a point where multiple signaling pathways converge, strategies for removing the inhibitory effects of IAPs seem to be potentially useful for overcoming the resistance of cancer cells to anticancer drugs (Deveraux and Reed, 1999b). Survivin, a member of the IAP family, is overexpressed in gliomas, and its presence marks a poor prognosis (Chakravarti *et al.*, 2002). Survivin blocks apoptosis by direct or indirect inhibition of downstream effector caspases (Zaffaroni and Daidone, 2002; Altieri, 2003). Recently, downregulation of not only survivin but also XIAP using siRNA has been reported to sensitize resistant melanoma cells to TRAIL-induced apoptosis (Chawla-Sarkar *et al.*, 2004). XIAP, one of the IAP family, is the most potent inhibitor of apoptosis that binds to and inhibits caspase-

3, -7, and -9 activities (Deveraux and Reed, 1999b). XIAP was recently reported to be a nonredundant modulator of TRAIL-mediated apoptosis in human cancer cells, providing a rationale for XIAP as a therapeutic target (Cummins *et al.*, 2004). Previously, Zhang *et al.* (2001) reported that XIAP inhibits the autocatalytic step of caspase-3 processing by association with p20 intermediate fragment of caspase-3 following treatment with TRAIL. In our study, TRAIL also induced partial proteolytic processing of procaspase-3 into the p20 fragment without any further processing into its active subunits both in U87MG and A172 cells, which are resistant to TRAIL-induced apoptosis. However, combined treatment with rottlerin and TRAIL recovered the complete proteolytic processing of caspase-3. Thus, downregulation of IAP proteins by rottlerin may contribute to apoptosis by removing the barriers blocking caspase activation. Recently, we showed that inhibition of Cdc2 by roscovitine enhanced TRAIL-induced apoptosis of glioma cells through transcriptional downregulation of XIAP expression and post-transcriptional downregulation of survivin expression via the proteasome pathway (Kim *et al.*, 2004). Polyubiquitination of survivin and proteasome-dependent destruction has been demonstrated in interphase cells, and mitotic phosphorylation of survivin on Thr34 by Cdc2-cyclin B1 has been associated with increased protein stability at metaphase (Zhao *et al.*, 2000; O'Connor *et al.*, 2002). Here, we found that rottlerin inhibited Cdc2 activity through downregulation of cyclin A, cyclin B, and Cdc2 in a dose- and time-dependent manner. In parallel with the downregulation of Cdc2, cyclin A, and cyclin B following treatment with rottlerin, both survivin and XIAP protein levels were rapidly and dramatically reduced. The functional significance of downregulation of Cdc2 kinase activity and the subsequent suppression of survivin and XIAP expression in rottlerin-enhanced TRAIL-induced apoptosis was confirmed by our observations that forced coexpression of Cdc2 with cyclin B could attenuate rottlerin-potentiated TRAIL-induced apoptosis, alleviating rottlerin-mediated downregulation of survivin and XIAP. Furthermore, overexpression of survivin or XIAP reduced the cell death induced by the combined treatment. Thus, the sensitizing effect of rottlerin on TRAIL-induced apoptosis may result from the downregulation of survivin or XIAP protein levels via rottlerin-mediated inhibition of Cdc2 activity.

In our study, combined treatment with rottlerin and TRAIL was cytotoxic to glioma cells, while normal astrocytes were spared. Furthermore, this combined treatment demonstrated considerable cytotoxicity in glioma cells overexpressing Bcl-2 or Bcl-xL, which are generally resistant to many other chemotherapeutic treatments. Therefore, treatment with TRAIL in combination with subtoxic doses of rottlerin suggests a potential applicability for treating resistant gliomas, although extensive animal studies and preclinical trials using this regimen will be required.

Materials and methods

Chemicals and antibodies

Recombinant human TRAIL/Apo2 ligand was from KOMA Biotech Inc. (Korea) or from Alexis Biochemicals (Coger, Paris, France). Calcein-AM and Etd-1 were from Molecular Probe (Eugen, OR, USA). Rottlerin, z-VAD-fmk, z-IETD-fmk, z-DEVD-fmk, and z-LEHD-fmk, pepstatin A, and PD150606 were from Calbiochem (San Diego, CA, USA). CA-074-Me was from Peptide Research Institute (Osaka, Japan). The following antibodies were used: anti-caspase-8, caspase-3, caspase-7, survivin, FLIP, and XIAP (Stressgen, British Columbia, Canada); anti-caspase-9, caspase-2, Rb, FAK, Bcl-2, Bcl-xL, c-IAP2, cyclin A, cyclin B, Cdc2, Myc, and PKC δ (Santa Cruz Biotechnologies, Santa Cruz, CA, USA); anti-phospho-Akt, Akt, phospho-p38, phospho-JNK, p38, JNK, and Bid (Cell Signaling, Beverly, MA, USA); anti-Flag M2 (Sigma, St Louis, MO, USA); anti-CrmA, DR4, and PKC δ (BD Pharmingen, San Diego, CA, USA); anti- α -tubulin and DR5 (Calbiochem, San Diego, CA, USA); anti-PARP (Upstate biotechnology, Lake Placid, NY, USA); horseradish peroxidase-conjugated anti-rabbit IgG and horseradish peroxidase-conjugated anti-mouse IgG HRP (Zymed Laboratories Inc., South San Francisco, CA, USA).

Culture of glioma cell lines and normal human astrocytes

The human malignant glioma cell lines U87MG, A172, T98, and U251MG were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and antibiotics (GIBCO-BRL, Life Technologies, Grand Island, NY, USA). The primary cultures of normal human astrocytes were prepared from 14-week-gestation fetal cerebrum tissues as described previously (Kim, 1985; Kim *et al.*, 1986). Human astrocyte cultures were subcultured in DMEM containing 10% fetal bovine serum every 2 weeks and cell cultures passage number less than 5 were used in the present study. Immunofluorescence study indicated that better than 99% of cells expressed glial fibrillary acidic protein-positive immunoreactivity, a cell type-specific marker for astrocytes. Permission to use human brain tissues for research was granted by the Ethics Committee of the University.

Measurement of cellular viability

Cell viability was assessed by double labeling of cells with 2 μ M calcein-AM and 4 μ M Etd-1. The calcein-positive live cells and Etd-1-positive dead cells were visualized using a fluorescence microscope (Nikon Diaphot 300, Japan).

Immunoblotting

Cells were washed in phosphate-buffered saline (PBS) and lysed in boiling sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (62.5 mmol/l Tris (pH 6.8), 1% SDS, 10% glycerol, and 5% β -mercaptoethanol). The lysates were boiled for 5 min, separated by SDS–PAGE, and transferred to an Immobilon membrane (Millipore, Bedford, MA, USA). After blocking nonspecific binding sites for 1 h by 5% skim milk, membranes were incubated for 2 h with specific Abs. Membranes were then washed three times with TBST and incubated further for 1 h with horseradish peroxidase-conjugated anti-rabbit, -mouse, or -goat antibody. Visualization of protein bands was accomplished using ECL (Amersham Life Science, Buckinghamshire, UK).

Expression of WT PKC δ , DN PKC δ mutant, Cdc2, cyclin A, or cyclin B by transient transfection

U87MG cells were plated into 24-well plates at 5×10^4 cells/well. After 24 h, cells were transfected with the plasmid encoding WT PKC δ , dominant-negative (DN PKC δ mutant (PKC δ K376R) (generously gifted by Dr JF Mushinski, NIH), WT Cdc2 (kindly provided by Dr Sander van den Heuvel, Massachusetts General Hospital), cyclin A, or cyclin B (kindly provided by Dr Paul Robbins, University of Pittsburgh) at the indicated concentrations using Lipofectamine Plus reagent (GIBCO-BRL, Life Technologies, Grand Island, NY, USA) following the manufacturer's instructions. Transfected cells were incubated for 24 h and then further treated with 10 μ M rottlerin and/or 100 ng/ml TRAIL for 24 h. Transfection efficiency, assessed using a green fluorescence protein-encoding plasmid, reached about 70% in these experiments. Cellular viability was assessed using calcein-AM and Etd-1 as described above.

PKC δ immune complex kinase assay

U87MG cells (5×10^5 cells) were plated into 60 mm dish. After 24 h, cells were transfected with expression vectors encoding WT or DN PKC δ at the indicated concentrations. Cells were washed twice with PBS and incubated in lysis buffer (20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin). Insoluble materials were removed by centrifugation (13 000 r.p.m. for 10 min at 4°C), and protein concentrations were quantitated by Bio-Rad protein assay. For immunoprecipitation experiments, 100 μ g of protein was incubated with anti-PKC δ antibody (1 μ g/reaction, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for 3 h and further incubated with 15 μ l of protein A–agarose beads (Oncogene Research Products, Cambridge, MA, USA) for 2 h at 4°C. Immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (20 mM Tris (pH 7.5), 20 mM MgCl₂, 1 mM CaCl₂). A PKC δ kinase assay on myelin basic protein (MBP) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was performed by mixing the respective immune complexes with 20 μ g/ml MBP, 10 μ M ATP, 50 μ g/ml phosphatidyl serine, and 5 μ Ci of [γ -³²P]ATP in 40 μ l of kinase buffer. The kinase reaction was performed at 30°C for 30 min and then terminated by the addition of 2 \times SDS–PAGE sample buffer. The reaction mixtures were resolved by SDS–PAGE, the dried gel was exposed to film, and the substrate phosphorylation was assessed by autoradiography.

Cdc2 immune complex kinase assay

After treatment of U87MG cells with rottlerin and/or TRAIL as indicated, cells were lysed in buffer A (1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 20 mM NaF, 1 mM Na₃VO₄, 10 μ g/ml PMSF) at 4°C for 15 min. Cell lysates were cleared by centrifugation at 13 000 r.p.m. for 15 min. Protein concentrations were quantitated by Bio-Rad protein assay. A total of 500 μ g of protein was used for each immunoprecipitation. Cdc2 in cell extracts was incubated with anti-Cdc2 antibody (1 μ g/reaction) for 3 h at 4°C. A measure of 15 μ l of protein A/G–agarose (Oncogene Research Products, Cambridge, MA, USA) was added into the mixture, which was then further incubated for 1 h. Immune complexes were centrifuged at 2500 r.p.m. for 5 min and the precipitates were washed three times with buffer A and twice with kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT). Cdc2 kinase assay on histone H1 was performed by mixing the respective immune

complexes with 5 μ g of histone H1 and 10 μ Ci of [γ - 32 P]ATP in 30 μ l of kinase buffer. The kinase reaction was performed at 30°C for 30 min and then terminated with 2 \times SDS-PAGE sample buffer. The reaction mixtures were resolved by SDS-PAGE analysis. Gels were stained with Coomassie Blue staining solution and dried. The extent of phosphorylation was measured by liquid scintillation counting of the gel slices of each substrate.

Establishment of the stable U87MG cell lines overexpressing CrmA, Bcl-2, Bcl-xL, XIAP, survivin, or constitutively active Akt

U87MG cells were transfected with the following: a mammalian expression vector containing CrmA cDNA (Tewari *et al.*, 1995) (kindly provided by Professor VM Dixit, University of Michigan Medical School); vectors containing Flag-tagged *bcl-2* cDNA and *bcl-xL* (Huang *et al.*, 1997) (kindly provided by Dr A Strasser, The Walter and Eliza Hall Institute of Medical Research, Australia); a vector containing Myc-tagged XIAP (Nomura *et al.*, 2003) (kindly provided by Professor T Nomura, Oita Medical University, Japan); or a vector containing Myc-tagged active Akt (Upstate Biotechnology, NY, USA). Stable cell lines overexpressing Bcl-2 or Bcl-xL were selected in fresh media containing puromycin (4 μ g/ml). Overexpression of Bcl-2 or Bcl-xL in the stable cell lines was analysed by Western blotting using anti-Flag antibody. Stable cell lines overexpressing CrmA, active Akt or XIAP were selected in fresh media containing G418 (500 μ g/ml). Overexpression of CrmA in the stable cell lines was analysed by Western blotting using anti-CrmA antibody, while overexpression of XIAP or active Akt in the respective stable cell lines was analysed using anti-c-Myc antibody (9E10). To establish stable cell lines overexpressing survivin, U87MG cells were cotransfected with a flag/survivin fusion protein expression

vector (Kobayashi *et al.*, 1999) (kindly provided by Professor T Tokuhisa, Chiba University Graduate School of Medicine, Japan) and pcDNA3. Transfected cells were selected with fresh media containing G418 (500 μ g/ml) and overexpression of survivin was analysed by Western blotting using anti-Flag antibody.

Abbreviations

TRAIL, TNF-related apoptosis-inducing ligand; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DN, dominant negative; FLIP, FLICE-inhibitory protein; IAP, inhibitor of apoptosis; XIAP, X-chromosome-linked IAP.

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