Enhanced susceptibility to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in oral squamous cell carcinoma cells treated with phosphatidylinositol 3-kinase inhibitors

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Abstract. In general, oral squamous cell carcinoma (OSCC) cells are relatively resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis during culture in vitro. Here, we studied the role of phosphatidylinositol 3-kinase (PI 3-K)/Akt in survival and apoptosis of these cells. The PI 3-K inhibitors wortmannin and LY294002 markedly suppressed phosphorylation of Akt and accelerated TRAIL-mediated apoptosis in OSCC cells. Addition of TRAIL to PI 3-K inhibitor-treated cells resulted in caspase-8 activation and loss of mitochondrial membrane potential. Furthermore, inhibitors of caspase-3, -8 and -9 reduced the accelerative effect of PI 3-K inhibitors on TRAIL-mediated apoptosis. These results suggest that the pro-apoptotic effect of PI 3-K inhibitors on TRAIL-mediated apoptosis may contribute to both the extrinsic and intrinsic pathways. Although PI 3-K inhibitors did not affect expression of the TRAIL receptors DR4 and DR5, we observed a marked reduction in expression of cellular FLICE-inhibitory protein (c-FLIP), Bcl-2, cellular inhibitor of apoptosis protein-1 (cIAP-1) and X-linked IAP (XIAP), whereas Bax was up-regulated and no significant difference was observed in expression of Bcl-xl, Bak or cIAP-2. Therefore, the PI 3-K/Akt signaling pathway may represent a novel strategy for overcoming resistance to TRAIL-mediated apoptosis in OSCC cells.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that was identified originally through sequence homology to Fas ligand (FasL) and TNF (1,2). TRAIL can induce apoptosis in a variety of tumor cells by engaging the death receptors DR4 and DR5, resulting in activation of the extrinsic apoptotic pathway (3,4). In the extrinsic pathway, receptor-mediated activation of caspase-8 and -10 is followed by activation of caspase-3, which cleaves intracellular substrates leading to cell death. The intrinsic or mitochondrial pathway is linked to the extrinsic pathway through activated caspase-8, which cleaves the pro-apoptotic Bcl-2 family member Bid, producing a truncated fragment that associates with Bax. The Bid/Bax complex induces mitochondrial release of cytochrome c, formation of the apoptosome and activation of caspase-9, followed by activation of executioner caspase-3, -6 and -7, which in turn promote further cleavage of intracellular proteins and cell death (5).

Many tumor cells, including the majority of squamous cell carcinoma (SCC) cells, also express DR4 and DR5 on their surfaces (6). TRAIL receptors are expressed in a variety of cancer and transformed cells, as well as in normal cells, where they have little effect (7). However, the response to TRAIL can be highly variable and resistance is observed in many types of cancer (5,8). The initiation of apoptosis is controlled by integration of both pro- and anti-apoptotic signal transduction pathways, which mediate stimulation including the signaling via death receptors (9). Although the TRAIL death pathway is well defined, no dominant mechanism has yet been identified for resistance to TRAIL-induced cell death. Several intracellular anti-apoptotic molecules, such as members of the anti-apoptotic Bcl-2 and inhibitor of apoptosis protein (IAP) families, as well as cellular FLICE-inhibitory protein (c-FLIP), have been shown to inhibit the apoptotic signaling cascade via inhibition of mitochondrial cytochrome c release, apoptosome formation and recruitment of procaspase-8 to the death receptor domain (10-13). In addition, death receptor mutations and deficiencies in caspase-8 and Bax have been implicated in TRAIL resistance (4,13-17).
Although intracellular mediators of apoptosis are currently being investigated, they remain largely unknown. Phosphatidylinositol 3-kinase (PI 3-K) is known to control cell survival/death through phosphorylation of Akt in cancer cells (18-20). Activated Akt has been shown to play a role in growth factor- and cytokine-mediated survival, and it protects cells from apoptosis induced by a variety of stimuli (21). The PI 3-K/Akt signaling pathway also controls death receptor-mediated apoptosis in various cells, including cancer cells (22-24). However, the mechanism by which TRAIL-mediated apoptosis is attenuated via the PI 3-K/Akt signaling pathway remains unclear. Activated Akt phosphorylates Bad, procaspase-9, forkhead, cyclic AMP response element-binding protein and IκB kinase, resulting in suppression of the apoptotic signaling cascade and expression of anti-apoptotic genes (18-20). Furthermore, Akt has been shown to mediate down-regulation of the pro-apoptotic Bcl-2 family and up-regulation of the anti-apoptotic c-FLIP, IAPs and Bcl-2 family proteins (25-27).

Strategies to overcome these resistance mechanisms are under intensive investigation. In this study, we examined whether or not PI 3-K inhibitors can affect the susceptibility of oral SCC (OSCC) cells to TRAIL-mediated apoptosis. In addition, we considered their effect on the expression of c-FLIP, as well as Bcl-2 and IAP family proteins. We demonstrated that in OSCC cells PI 3-K inhibitors enhance TRAIL-mediated apoptosis through modulation of c-FLIP, Bcl-2, Bax, cellular IAP (cIAP)-1 and X-linked IAP (XIAP).

Materials and methods

Reagents. DMEM and FBS were obtained from Gibco-BRL (Gaithersburg, MD). Recombinant human soluble TRAIL, anti-DR4, anti-DR5, and the MEBCYTO apoptosis kit (employing FITC-conjugated annexin V) were purchased from MBL (Nagoya, Japan). Mouse anti-FLIP monoclonal antibody (mAb) was obtained from Alexis Corp. (Lausanne, Switzerland). We used mAbs against the following: Bax, cIAP-1, cIAP-2, XIAP and active caspase-8 (R&D Systems Inc., Minneapolis, MN); Bak (Stressgen Biotechnology, Victoria, Canada); and Bcl-2, Bcl-xL, Akt and phospho-Akt (Ser 473) (Santa Cruz Biotechnology, Santa Cruz, CA). Control antibodies were obtained from Pharmingen (San Diego, CA). We used caspase inhibitors against the following: caspase family (VAD-FMK); caspase-3 (DEVD-FMK); caspase-8 (IETD-FMK); caspase-9 (LEHD-FMK); and FMK negative control (FA-FMK; Trevigen Inc., Gaithersburg, MD). The PI 3-K inhibitors wortmannin and LY 294002 were obtained from Calbiochem (San Diego, CA) and Cayman Chemical (Ann Arbor, MI), respectively. All other chemicals were of analytical grade.

Cell lines and culture conditions. The human OSCC lines HSC-2 and NA were grown as adherent monolayers. HSC-2 and NA are cell lines established from SCC of the oral cavity. Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO₂. Neither wortmannin (500 nM) nor LY294002 (30 μM) had any effect on cell viability under the conditions indicated.

Detection of TRAIL-mediated apoptosis. Annexin V-FITC binding was used as a sensitive method for measuring apoptosis and was performed as described previously, with some modifications (28,29). OSCC cells (1x10⁷ cells/ml) were preincubated with or without PI 3-K inhibitor for 2 h. Treated OSCC cells (1x10⁶ cells/ml) were exposed to TRAIL (200 ng/ml) for 24 h, then harvested and incubated for 15 min at room temperature in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) containing a saturating concentration of annexin V-FITC, according to the manufacturer's protocol. After incubation, cells were pelleted and analyzed using a FACScan flow cytometer and CellQuest software (Becton-Dickinson Co., Mountain View, CA). A subset of TRAIL-mediated apoptosis experiments were performed in the presence of caspase inhibitors (20 μM).

Mitochondrial membrane potential measurement. Mitochondrial membrane potential determination was performed using the MitoLight apoptosis detection kit (Chemicon Inc., Temecula, CA), which contains the lipophilic mitochondrial activity marker MitoLight. In brief, OSCC cells (1x10⁷ cells/ml) were preincubated with or without PI 3-K inhibitor for 2 h. Treated cells (1x10⁶ cells/ml) were then exposed to TRAIL (200 ng/ml), for 12 h and harvested. Cells were incubated in MitoLight solution for 20 min, then pelleted and analyzed using a FACScan flow cytometer and CellQuest software (Becton-Dickinson Co.).

Flow cytometry analysis of TRAIL receptor expression. Cell surface expression of TRAIL receptor was determined using indirect immunofluorescence analysis. OSCC cells (1x10⁷ cells/ml) were incubated with wortmannin (500 nM) or LY294002 (30 μM) for 6 h, after which these cells were treated with a saturating concentration of anti-DR4 or anti-DR5 mAb for 40 min. After washing twice with PBS, cells were incubated for 30 min at 4°C with anti-mouse IgG-FITC-conjugated secondary Ab dissolved in PBS containing 0.1% BSA and 0.1% sodium azide. Cells were washed again with PBS and fixed with 1% paraformaldehyde in PBS. Simultaneous negative control staining was performed without primary Ab. Stained cells were analyzed using a FACScan flow cytometer with CellQuest software (Becton-Dickinson Co.).
Western blot analysis. Western blot analysis was used to detect expression of Akt, p-Akt, c-FLIP, Bax, Bak, Bcl-2, Bcl-xL, cIAP-1, cIAP-2 and XIAP. OSCC cells (1x10^7 cells/ml) were incubated with wortmannin (500 nM) or LY294002 (30 μM) for 1 h for detection of Akt and p-Akt, and for 6 h for detection of c-FLIP, Bax, Bak, Bcl-2, Bcl-xL, cIAP-1, cIAP-2 and XIAP. In the caspase-8 experiments, PI 3-K treated cells were exposed to TRAIL (200 ng/ml) for 8 h and then harvested. After treatment, cells (1x10^7 cells/ml) were sedimented and disrupted in lysis buffer containing 50 mM

Figure 2. TRAIL-mediated apoptosis in HSC-2 and NA cells treated with PI 3-K inhibitors. Representative histograms of HSC-2 (A) or NA (B) cells treated with 500 nM wortmannin (lower panels, left and right) or 30 μM LY294002 (upper panels, left and right), then 200 ng/ml recombinant TRAIL (both panels, center and right). Dark and bright lines indicate untreated controls and treated experimental cells, respectively. Percentage of TRAIL-mediated apoptosis in treated HSC-2 (C) or NA (D) cells. Results represent the means ± SD of four independent experiments. *Statistically significant differences (p<0.05) between untreated and PI 3-K inhibitor- or TRAIL-treated cells.
Figure 3. TRAIL-mediated caspase-8 activation in HSC-2 and NA cells treated with PI 3-K inhibitors. HSC-2 (left panel) and NA (right panel) were treated with or without wortmannin (500 nM) or LY294002 (30 μM) for 2 h, then harvested and incubated with recombinant TRAIL (200 ng/ml) for 8 h. Caspase-8 activation was determined using Western blot analysis. All experiments were performed four times independently. Lane 1. untreated; lane 2. wortmannin; lane 3. LY294002. The relative abundance of activated caspase-8 in untreated and treated cells was normalized to that of β-actin. Untreated controls were set as 1.0.

Results

Effect of PI 3-K inhibitors on Akt phosphorylation in OSCC cells. To confirm that the PI 3-K inhibitors wortmannin and LY294002 reduce activation of Akt in HSC-2 and NA cells, we examined Akt phosphorylation. It is likely that Akt is constitutively activated in OSCC cells, as untreated cells exhibited Akt phosphorylation (Fig. 1). Treatment with both PI 3-K inhibitors (500 nM wortmannin and 30 μM LY294002) effected down-regulation of Akt phosphorylation (Fig. 1), suggesting that PI 3-K inhibitors can modulate cell survival pathways in OSCC cells.

Effects of PI 3-K inhibitors on TRAIL-mediated apoptosis. We examined the annexin V binding of HSC-2 and NA cells treated with PI 3-K inhibitors, in order to determine whether or not they exhibited altered susceptibility to TRAIL-mediated apoptosis. Untreated HSC-2 and NA cells were relatively resistant to TRAIL-mediated apoptosis, whereas apoptosis levels were significantly higher in PI 3-K inhibitor-treated cells (Fig. 2). Spontaneous apoptosis did not appear to be elevated by either of the PI 3-K inhibitor treatments (Fig. 2).

Statistical analysis. The values are presented as the means ± SD. Data were analyzed using a one-way repeated measure ANOVA. p<0.05 was considered to be statistically significant.
Effect of PI 3-K inhibitors on TRAIL-induced apoptosis of OSCC cells (Fig. 5). Thus, the pro-apoptotic activity of PI 3-K inhibitors is related to both the extrinsic and intrinsic pathways within the caspase cascade.

Effect of PI 3-K inhibitors on TRAIL receptor expression. To investigate whether or not the expression of TRAIL receptors was altered by treatment with PI 3-K inhibitors, we examined expression of DR4 and DR5 using a FACScan flow cytometer. TRAIL induces apoptosis by binding either the DR4 or DR5 receptor, resulting in activation of the extrinsic pathway (4,13). Both DR4 and DR5 were expressed constitutively and we observed no significant difference following addition of either wortmannin or LY294002 (Fig. 6). Therefore, these data indicate that the acceleration of TRAIL-mediated apoptosis observed with PI 3-K inhibitor treatment was not a consequence of increased TRAIL receptor expression.

Effect of PI 3-K inhibitors on c-FLIP expression. Overexpression of c-FLIP is an established mechanism for resistance to TRAIL-mediated apoptosis (4,13). Following treatment with PI 3-K inhibitors, we examined the level of c-FLIP expression using Western blot analysis. c-FLIP was expressed constitutively in both HSC-2 and NA cells, whereas following treatment with PI 3-K inhibitors there was a marked decrease in its expression (Fig. 7). These results suggest that in OSCC cells, the PI 3-K/Akt signaling pathway can modulate TRAIL-mediated apoptosis via expression of c-FLIP.

Effect of PI 3-K inhibitors on expression of Bcl-2 family proteins. Members of the Bcl-2 family are important factors in the modulation of TRAIL-mediated apoptosis (4,13). Therefore, we examined the effect of PI 3-K inhibitors on expression of Bak, Bax, Bcl-2 and Bcl-xL in HSC-2 cells. Treatment with both wortmannin and LY294002 increased expression levels of the pro-apoptotic protein Bax and downregulated the anti-apoptotic protein Bcl-2 (Fig. 8). In contrast, expression of the anti-apoptotic protein Bcl-xL and the pro-apoptotic protein Bak were unaffected by this treatment (Fig. 8). These results suggest that in OSCC cells, the PI 3-K/Akt signaling pathway may modulate TRAIL-mediated apoptosis via regulation of Bcl-2 family proteins such as Bax and Bcl-2.

Effect of PI 3-K inhibitors on expression of IAP family proteins. Members of the IAP family also contribute to
susceptibility to TRAIL-mediated apoptosis (4,13) and we examined the effect of PI 3-K inhibitors on expression of cIAP-1, cIAP-2 and XIAP in HSC-2 cells. Treatment with both wortmannin and LY294002 down-regulated expression of cIAP-1 and XIAP, but did not alter expression of cIAP-2 (Fig. 9). These results suggest that in OSCC cells, the PI 3-K/Akt signaling pathway may also modulate TRAIL-mediated apoptosis via regulation of IAP family proteins such as cIAP-1 and XIAP.

Discussion

PI 3-K/Akt suppresses apoptosis mediated by both ligation of death receptors and the mitochondrial pathway (18,22-24). Treatment with PI 3-K inhibitors (wortmannin and LY294002) caused marked suppression of Akt phosphorylation in the OSCC cell lines HSC-2 and NA (Fig. 1). These results suggest that the PI 3-K/Akt pathway plays a role in OSCC cell survival and that PI 3-K inhibitors can induce apoptosis in these cells. PI 3-K inhibitors such as wortmannin and LY294002 are known to possess anti-tumor activities (30,31) and previous studies have indicated that the PI 3-K/Akt signaling pathway...
can modulate TRAIL-mediated apoptosis in cancer cells (22-24,32,33). Therefore, we used HSC-2 and NA cell lines, which are resistant to TRAIL-induced apoptosis, to examine the effect of treatment with PI 3-K inhibitors on susceptibility to apoptosis via this process. We demonstrated that in OSCC cells, TRAIL-mediated apoptosis is significantly enhanced by treatment with PI 3-K inhibitors (Fig. 2) and that this treatment results in activation of caspase-8 (Fig. 3) and loss of mitochondrial membrane potential (Fig. 4).

Next, we investigated the effect of various caspase inhibitors on the enhancement of TRAIL-induced apoptosis by PI 3-K inhibitors and observed that inhibitors of caspase-8, -9 reduced the effect of PI 3-K inhibitors (Fig. 5). These results suggest that in PI 3-K inhibitor-treated OSCC cells, TRAIL-induced apoptosis is related to both the extrinsic and intrinsic pathways.

Up-regulation of TRAIL receptor expression may provide an explanation for the amplification of TRAIL-induced apoptosis effected by PI 3-K inhibitors. Previous studies have shown that in cancer cells, expression of TRAIL receptors is modulated by extracellular stimuli such as anti-cancer drugs, radiation and cytokines, all of which amplify cellular susceptibility to TRAIL-mediated apoptosis (6,34-36). In contrast, other studies have shown that TRAIL receptor expression is not altered by pro- or anti-apoptotic stimuli (27,33,37). Thus, we examined whether or not DR4 or DR5 expression was altered by treatment with PI 3-K inhibitors and observed no significant change (Fig. 6), confirming the results of earlier studies (22-24). Interestingly, other studies have also demonstrated that DR4 and DR5 expression levels do not correlate with susceptibility to TRAIL-induced apoptosis (37,38). These results also suggest that the pro-apoptotic activity of PI 3-K inhibitors do not contribute to expression of DR4 or DR5.

c-FLIP is a proximal regulator during TRAIL-mediated apoptosis (4,10,13) and high levels of this protein have been related to resistance to TRAIL-mediated apoptosis (4,10,13,39). c-FLIP is a competitive inhibitor of caspase-8 for binding to the TRAIL receptor complex and thus, its activity down-regulates the downstream TRAIL signaling pathway (4,13,39). In cancer cells, c-FLIP expression levels are higher than in normal tissue and these elevated levels allow the cells to overcome TRAIL-mediated apoptosis (40,41). Furthermore, recent studies, including our previous investigations, have indicated that transfection of small interfering RNA or antisense oligonucleotides against c-FLIP enhanced death receptor-induced apoptosis in various cancer cells (28,42,43). As caspase-8 inhibitors suppressed increased TRAIL-mediated apoptosis caused by PI 3-K inhibitors, the pro-apoptotic effect of the latter group may act upstream of caspase-8 activation.

Elevated Akt activity up-regulates c-FLIP and inhibits TRAIL-mediated apoptosis in various cancer cells (22,23,42,44) and PI 3-K inhibitors down-regulate the expression of c-FLIP in cancer cells (27,29). Thus, we investigated c-FLIP expression in PI 3-K inhibitor-treated OSCC cells and observed that c-FLIP was strongly down-regulated by both wortmannin and LY294002 (Fig. 7). Therefore, down-regulation of c-FLIP expression represents a pro-apoptotic factor in the effects of PI 3-K inhibitor on TRAIL-mediated apoptosis.

In this study, mitochondrial membrane potential was lost following addition of TRAIL to PI 3-K inhibitor-treated OSCC cells and the caspase-9 inhibitor suppressed this process. These results indicate that the pro-apoptotic activity of PI 3-K inhibitors is related to the intrinsic pathway. Previously, we have shown that wortmannin and LY294002 also inhibited Bad phosphorylation in OSCC cells (29); phosphorylated Bad is known to bind mitochondrial Bcl-2 or Bcl-XL, preventing cytochrome c release and caspase-9 activation (45). Previous reports have also suggested that Akt-related TRAIL resistance is mediated via inhibition of Bid cleavage and that the pro-apoptotic activity of PI 3-K inhibitors is linked to the intrinsic pathway (22,32). The intrinsic pathway is regulated by pro- and anti-apoptotic proteins which include members of the Bcl-2 and IAP families and TRAIL resistance can be mediated at the mitochondrial level by Bcl-2 and Bcl-XL (4,13,46). The IAP family of caspase inhibitors act predominantly as downstream inhibitors of TRAIL pathways; they are expressed at high levels in various cancer cells, suggesting that they play a key role in preventing apoptosis (4,13,47). Here we have shown that in OSCC cells, PI 3-K inhibitors down-regulate the anti-apoptotic proteins Bcl-2, cIAP-1 and XIAP, and up-regulate the pro-apoptotic protein Bax. Thus, the PI 3-K inhibitors may enhance TRAIL-mediated apoptosis in OSCC cells by modulation of apoptosis-regulated protein levels. Although previous studies have shown that PI 3-K inhibitors regulate expression of various members of the Bcl-2 and IAP families, these results do not necessary hold across different cancer cell types (22,23,32). In order to elucidate fully the role played by these families in TRAIL-mediated apoptosis of different cancer cell types, it will be necessary to perform knockdown and overexpression analyses. In cancer cells, Akt is activated constitutively, promoting cellular survival and resistance to chemotherapy, ionizing radiation, Fas and TRAIL (23,48). However, anti-cancer drugs and radiation suppress PI 3-K/Akt activation, inducing cell death (49,50). Recent reports have demonstrated that anti-cancer drugs and PI 3-K inhibitors can act synergistically in malignant tumors (51,52). This suggests that a combined approach may be effective for activating death receptors and TRAIL-dependent apoptosis in OSCC cells.

In conclusion, we found that PI 3-K/Akt inactivation induced TRAIL-mediated apoptosis in OSCC cells via down-regulation of c-FLIP, Bcl-2, cIAP-1 and XIAP, and up-regulation of Bax. Our results therefore, support the hypothesis that targeting of the PI 3-K/Akt signaling pathway is a powerful strategy for overcoming OSCC cell resistance to conventional therapeutic treatments. As such, pharmacological inhibitors of the PI 3-K/Akt signaling pathway might achieve this aim: recent studies have demonstrated that agonistic mAbs against DR4 and DR5 can induce apoptosis in a variety of cultured and primary cancer cells (53). Other studies have shown that the combination of anti-human DR4 and DR5 mAbs with chemotherapeutic drugs or radiotherapy synergistically enhanced anti-tumor effects (54,55). Given the inherent difficulties of using wortmannin and LY294002 (stability, solubility and toxicity), efforts are underway to develop new PI 3-K pathway inhibitors; recently, a novel candidate has been reported that exhibits strong anti-tumor activity against human cancers without concomitant toxic effects on organs (56).
References


