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Letter to the Editor

Targeting Bcl-x_L in esophageal squamous cancer to sensitize to chemotherapy plus TRAIL-induced apoptosis while normal epithelial cells are protected by blockade of caspase 9

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Dear Editor,

TRAIL induces apoptotic cell death upon binding to either of two proapoptotic TRAIL receptors, TRAIL R1 (DR4)[1] or TRAIL R2 (KILLER/DR5).[2] Activation of the proapoptotic death receptors by TRAIL engagement induces the formation of a death-inducing signaling complex (DISC), which consists of receptor, FADD, as an adaptor,[3] and caspase 8 as an initiator caspase. Once the DISC is formed, the caspase 8 is autoprocessed and activated by induced proximity.[4] In FAS/FASL-mediated apoptosis, two types of apoptotic signaling pathways[5] exist, namely type I and type II. In type I cells, the activated caspase 8 could directly activate executioner caspase 3 without the involvement of the mitochondrial pathway. Even though either Bcl-2 or Bcl-x_L may be overexpressed, the apoptotic signaling is not blocked in type I cells. In type II cells, there is little caspase 8 recruitment into the DISC; not enough to efficiently activate caspase 3, but enough to activate the mitochondrial pathway. Bid plays an important role in bridging signals from death receptors (extrinsic pathway) to the mitochondria (intrinsic pathway). Activated caspase 8 cleaves Bid and then, truncated Bid (tBid) translocates into mitochondria followed by Bax translocation, BAK oligomerization, cytochrome *c* release, and activation of caspase 9 and the other downstream caspases.[6,7] The overexpression of either Bcl-2 or Bcl-x_L could completely block the apoptotic signaling in type II cells. The observations that Bid is cleaved in TRAIL-mediated apoptosis,[8] that some Bcl-2- or Bcl-x_L-overexpressing cells are still sensitive to TRAIL,[9] and that in the presence of z-LEHD-FMK, a caspase 9 inhibitor, caspase 3 is activated in some cancer cells but not in other cancer cells[10] suggest that the same types of pathways (type I and type II) may be utilized in TRAIL-mediated apoptosis. TRAIL is a promising agent for development as a cancer therapeutic because it appears to specifically kill transformed and cancer cells, whereas most normal cells appear to be resistant to TRAIL.[11] Systemic administration of TRAIL is safe in mice and can kill breast or colon-xenografted tumors and prolong survival.[11] However, the potential utility and safety of systemic administration of TRAIL has been questioned recently because of results showing sensitivity of human but not monkey or mouse hepatocytes to recombinant human TRAIL *in vitro*.[12] Recently, we proposed the use of a specific caspase 9 inhibitor to circumvent that side effect based on the fact that hepatocytes are type II cells, and the inhibitor could rescue TRAIL hepatocytotoxicity without affecting the TRAIL killing effect towards cancer cells, many of which use a type I pathway in apoptotic signaling.[10] Although TRAIL could specifically kill cancer cells, there are still many cancer cells that are TRAIL resistant.[13] A number of mechanisms of TRAIL resistance have been described. For example, low or undetectable expression of DR4[13] or overexpression of TRAIL-decoy receptors, such as TRID (DcR1 or TRAIL-R3)[14] or TRUNDD (DcR2, or TRAIL-R4),[15] could block TRAIL-mediated apoptotic signaling. Loss of caspase 8 expression[16] or high expression of c-FLIP[13,17] make cancer cells also resistant to TRAIL. At the mitochondrial level, high expression of antiapoptotic Bcl-2 family proteins such as Bcl-2 or Bcl-x_L might block death signal propagation if the cells use a type II pathway.[5] Loss of expression of Apaf-1 documented in malignant melanoma could also provide a possible mechanism for TRAIL resistance at a postmitochondrial level.[18] It was additionally reported that elevated AKT activity conferred TRAIL resistance on LnCap, a prostate cancer cell line, in a tissue-specific manner.[19]

Little is known about the effects of TRAIL on the human gastrointestinal epithelium. To gain a better understanding of TRAIL-mediated apoptosis, we chose for the present study human esophageal tissue, including eight squamous esophageal cancer cell lines and one normal primary esophageal epithelial cell. To assess TRAIL cytotoxicity towards squamous esophageal cancer cells, we performed the active-caspase 3 assay. With this assay, when more than 15% of cells revealed active-caspase 3 in a cell line, we defined the cell line as TRAIL sensitive. With this definition, three esophageal cancer cell lines (TE2, TE9, and TE12) were sensitive to TRAIL (Figure 1a). Cycloheximide reversed the TRAIL resistance in all cases. Previously, we reported that the expression levels of DR4 or c-FLIP were well correlated with TRAIL sensitivity.[13] To evaluate determinants for TRAIL sensitivity in esophageal cancer cell lines, we performed Western blot immunostaining for expression of proteins involved in mediating the death signal (Figure 1b). Proapoptotic death receptors were highly expressed in TRAIL-resistant cancer cells and antiapoptotic decoy receptors were

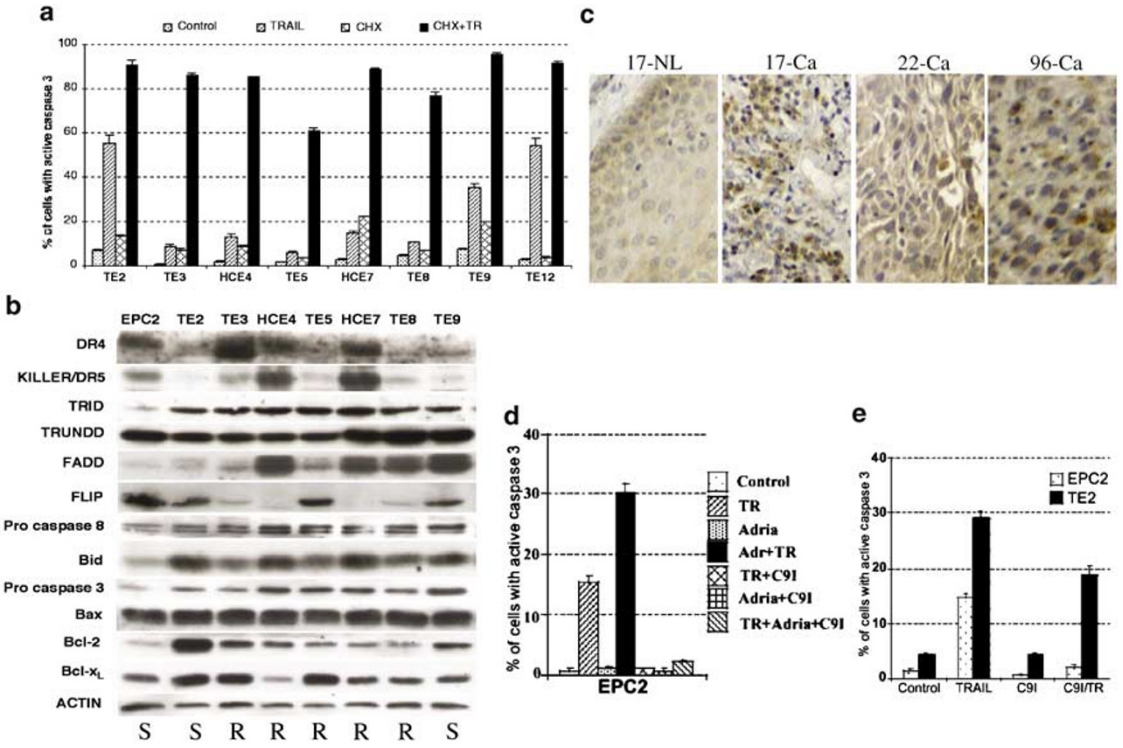

Figure 1 Analysis of determinants of TRAIL mediated cytotoxicity suggests sensitivity of primary esophageal epithelial cells as well as esophageal tumors, while there is some correlation between TRAIL resistance and overexpression of Bcl-x_L in TRAIL-resistant esophageal squamous cell carcinomas. (**a**) TRAIL cytotoxicity toward squamous esophageal cancer cells in the presence or absence of CHX. Each cancer cell line (5×10^5 cells/well) was seeded onto a six-well plate and the cells were treated with TRAIL crosslinked with anti-6X His Ab. After 16 h of TRAIL treatment, cells were harvested and an active-caspase 3 assay was performed. In case of CHX treatment, the cells were pretreated with CHX (10 μg/ml final concentration) for 6 h before the addition of TRAIL. We defined the cells as TRAIL sensitive when more than 30% of the cell population activated caspase 3. Experiments were performed in duplicate (mean value $\pm$ S.D.). Human esophageal cancer cell lines were previously described. All the esophageal cancer cell lines were grown with DMEM containing 10% FCS. Primary normal esophageal epithelial cells (EPC2) were established from normal human esophagus (unpublished data, Yasir Suliman, Oliver G Opitz and Anil K Rustgi). EPC2 cells have a diploid karyotype, and express E-cadherin as well as cytokeratins K4, K5, K13, and K14. EPC2 cells were grown in Keratinocyte-SFM medium (Gibco BRL, Rockville, MD, USA) containing bovine pituitary extract (40 μg/ml) and epidermal growth factor (1 ng/ml). For detecting apoptosis mediated by TRAIL, an active-caspase 3 assay was performed using the Cytofix/Cytoperm kit (Pharmingen, San Diego, CA, USA) as previously described. Briefly, 5×10^5 cells were seeded onto a six-well plate, after which, the cells were treated with TRAIL crosslinked with anti-6X His Ab. After 4 or 16 h of treatment, the cells were harvested, fixed, and then incubated with 0.125 μg/μl rabbit anti-active-caspase 3 Ab (Clone C92-605; Pharmingen) for 20 min in a dark room. After washing, the cells were probed with 0.125 μg/μl of the phycoerythrin (PE)-conjugated goat anti-rabbit secondary Ab (CALTAG Laboratories, Burlingame, CA, USA) for 20 min in a dark room. The intensity of PE was analyzed by flow cytometry using a Beckman–Coulter Epics Elite analyzer. The N-terminal histidine (His)-tagged recombinant human TRAIL (rhTRAIL, Thr-95 to Gly-281) was obtained from R&D Systems (Minneapolis, MN, USA). A mouse monoclonal anti-6X His antibody (R&D Systems) was used for crosslinking of the rhTRAIL. Cells were treated with 50 ng/ml final concentration of TRAIL and 1 μg/ml of anti-6X His Ab for 4 or 16 h. When the cells were treated with both cycloheximide (CHX) and TRAIL, CHX (10 μg/ml final concentration) was added 6 h before the addition of TRAIL. In the case of adriamycin, cells were pretreated with adriamycin (0.5 μM final concentration) for 16 h before the addition of TRAIL. (**b**) Relative expression level of signaling molecules involved in TRAIL-mediated apoptosis. Cell lysates were subjected to 12 or 15% SDS-PAGE, and then immunostained with Abs are indicated in the figure. TRAIL sensitivity was defined by the results of active-caspase 3 assay (see Figure 1); S = sensitive, R = resistant. Western blot analysis was carried out as previously described. Blotted membranes were immunostained with anti-DR4 (1 : 500; Pharmingen), anti-DR5 (1 : 500; IMGENEX, San Diego, CA, USA), anti-DcR1 (1 : 500; Pharmingen), anti-DcR2 (1 : 500; Pharmingen), anti-FADD (Clone IF7, 1 : 2000; Upstate Biotechnology, Lake Placid, NY, USA), anti-caspase 8 (Clone 5F7, 1 : 1000; Upstate Biotechnology), anti-FLIP (Clone Dave-2, 1 : 1000; Alexis Biochemicals, San Diego, CA, USA), anti-Bid (1 : 500; Pharmingen), anti-Bax (1 : 500, Pharmingen), anti Bcl-2 (Bcl-2/100, 1 : 500; Pharmingen), anti-Bcl-x_L (1 : 500; Pharmingen), anti-caspase 3 (E-8, 1 : 200; Santa Cruz, Santa Cruz, CA, USA), anti-PARP (1 : 2000; Roche Diagnostics GmbH, Mannheim, Germany), anti-TRAIL (1 : 500; PeproTech Inc., Rocky Hill, NJ, USA), or anti-actin (I-19, 1 : 200; Santa Cruz). (**c**) Bcl-x_L is overexpressed in human esophageal squamous cell carcinoma specimens. Bcl-x_L staining in normal human esophagus and three archival esophageal squamous cell carcinoma specimens. For the cancer (CA) case labeled #17, the normal esophageal tissue was obtained from the same patient. We used as primary antibody the mouse anti-human Bcl-x_L monoclonal antibody (Clone 2H12) obtained from Zymed (San Francisco, CA, USA) at a dilution of 1 : 50 in an overnight incubation at 4°C. (**d**) Active-caspase 3 assay for apoptosis after treatment with either TRAIL alone or TRAIL plus adriamycin (0.5 μM) in the presence or absence of z-LEHD-FMK on EPC2 normal esophageal epithelial cells. EPC2 (5×10^5) cells were treated with TRAIL (50 ng/ml) for 4 h. (**e**) Preferential cytoprotective effect of z-LEHD-FMK from TRAIL-mediated apoptosis in esophageal cells. Both EPC2-GFP (2×10^5 cells) and TRAIL-sensitive TE2 (5×10^5 cells) were grown in a six-well plate, and the cells were treated with TRAIL for 4 h in the presence or absence of z-LEHD-FMK. The active-caspase 3 assay was performed and the two populations of cells (EPC2-GFP and TE2) were discriminated by the presence or absence of green fluorescence. A bar graph is shown on the right to display the same result quantitatively. Experiments were performed in duplicate (mean value $\pm$ S.D.). The irreversible caspase inhibitors z-LEHD-FMK (caspase 9 inhibitor), z-IETD-FMK (caspase 8 inhibitor), and z-VAD-FMK (Pan caspase inhibitor) were obtained from R&D Systems. The caspase inhibitor was used at a final concentration of 20 μM and was added 2 h prior to the addition of TRAIL

expressed in every cancer cell line. Furthermore, TRID expression in EPC2 was lower than in the cancer cell lines. The expression level of FADD did not correlate with TRAIL sensitivity and c-FLIP, the inhibitor of caspase 8 activation, also showed no correlation with TRAIL sensitivity being undetectable on Western blot immunostaining in TRAIL-

resistant HCE4 and HCE7. c-FLIP was highly expressed in the TRAIL-sensitive TE2 and TE9 esophageal cancer cell lines. Procaspase 8 was expressed in all cell lines tested, and thus it does not appear to be silenced for example through hypermethylation. Bid was equivalently expressed and there was no difference among the cancer cell lines that could be correlated with TRAIL sensitivity or resistance. Antiapoptotic Bcl-2 or Bcl-x_L were variable in expression and TRAIL-sensitive TE2 and TE9 showed high-level expression of Bcl-2. However, the TRAIL-resistant cell lines TE3 and TE5 expressed high levels of Bcl-x_L. Proapoptotic Bax appeared constantly expressed among the cell lines. Taken together, in squamous esophageal cancers, the expression level of the molecules known to be involved in TRAIL-mediated apoptosis did not correlate universally with TRAIL sensitivity, although some TRAIL-resistant cell lines appeared to express high levels of Bcl-x_L. We found that Bcl-x_L was highly expressed in several archival esophageal squamous cell carcinomas tested (Figure 1c).

The fact that TRAIL has cytotoxicity toward normal human hepatocytes could hamper its use as a cancer therapeutic in humans. Recently, however, we reported that z-LEHD-FMK could rescue the normal human hepatocytes from TRAIL-mediated apoptosis.[10] Accordingly, we tested EPC2 for TRAIL sensitivity and EPC2 showed cytotoxicity in a dose-dependent manner (data not shown). When compared to the human foreskin keratinocyte (HFK), even at lower concentrations of TRAIL (5, 10, 25, or 50 ng/ml), EPC2 cells were sensitive to TRAIL while HFK were not affected (data not shown). We then tested the effect of the caspase 9 inhibitor z-LEHD-FMK on EPC2, and z-LEHD-FMK could completely protect the cells from TRAIL-mediated apoptosis even in the presence of adriamycin (Figure 1d). We also tested whether z-LEHD-FMK could protect TRAIL-sensitive esophageal cancer cells from apoptosis. We found that even in the presence of z-LEHD-FMK, TRAIL-sensitive esophageal cancer cells underwent apoptosis although the extent of apoptosis was slightly reduced (data not shown). We further confirmed this observation by incubating TRAIL in the presence or absence of z-LEHD-FMK with a mixed culture of EPC2-GFP and TRAIL-sensitive TE2. Active-caspase 3 assay results showed that EPC2-GFP cells (Figure 1e, C9I/TR) were almost completely protected from TRAIL-mediated apoptosis in the presence of z-LEHD-FMK, but TE2 (Figure 1e, C9I/TR) were still undergoing apoptosis. These results indicate that the combination of the caspase 9 inhibitor and TRAIL could be a useful strategy to circumvent undesirable cytotoxicity of TRAIL toward normal human cells or tissues as previously reported with normal human hepatocytes.[10]

Previously, we reported that the combination of adriamycin and TRAIL could induce apoptosis synergistically in TRAIL-resistant cancer cell lines irrespective of p53 status.[13] Accordingly, we tested adriamycin for whether the combination with TRAIL could induce synergistic cell killing in TRAIL-resistant esophageal cancer cells. When TRAIL was added to the TRAIL-resistant HCE4, TE3, and TE5 that were pretreated with adriamycin (0.5 μM) for 16 h, the cells underwent apoptosis and the effect was synergistic (Figure 2a; Adria + TRAIL/no inhibitor). We then checked the effect of z-LEHD-FMK on synergistic cell killing driven by the combination between adriamycin and TRAIL because z-LEHD-FMK is required to protect normal epithelial cells from killing by TRAIL. In case of HCE4, the cells were still killed in a synergistic way but in the cases of TE3 and TE5, the presence of z-LEHD-FMK almost completely blocked cell killing (Figure 2a; Adria + TRAIL/C9I). We also checked mitochondrial membrane potential ($\Delta\psi_m$) change after adriamycin treatment and we observed a drop of $\Delta\psi_m$ only in HCE4 cells (Figure 2b). Bax translocation from the cytosol to the mitochondria after adriamycin treatment also was observed only in the HCE4 cells (data not shown). According to these results, we speculated that the discrepancy might originate from the mitochondria and possible expression of Bcl-x_L. Therefore, we correlated the expression level of antiapoptotic Bcl-x_L and found a correlation between Bcl-x_L expression and sensitivity to adriamycin (Figure 2b and c). Thus, we tested another cell line, TE9, that showed low-basal expression of Bcl-x_L and the results were the same as those from HCE4 (data not shown, and Figure 2c). To confirm the effect of Bcl-x_L on the synergistic cell killing, we introduced Morpholino-modified antisense Bcl-x_L oligonucleotides (AS Bcl-x_L) into TE5. Upon introduction of AS Bcl-x_L, we observed the suppression of endogenous Bcl-x_L by Western immunostaining (Figure 2d inset), and this correlated with synergistic apoptotic cell death even in the presence of z-LEHD-FMK (Figure 2d), similar to what was observed in HCE4 or TE9 (data not shown). On the other hand, EPC2 was also synergistically killed by the combination of TRAIL and adriamycin, but it was almost completely protected from cell killing in the presence of z-LEHD-FMK (Figure 1d). Adriamycin did not induce a drop of $\Delta\psi_m$ in EPC2 (data not shown) and the expression level of Bcl-x_L is higher than that of HCE4 or TE9 (Figure 2c). Taken together, these results suggest that synergistic cell killing by the combination between adriamycin and TRAIL can be achieved in TRAIL-resistant cancer cells without inducing apoptosis in normal esophageal epithelial cells in the presence of z-LEHD-FMK. However, the success of the combined treatment may depend on the basal expression level of antiapoptotic Bcl-x_L.

We found that normal human esophageal epithelial cells are susceptible to TRAIL-mediated apoptosis even at low concentrations (5, 10, 25, or 50 ng/ml) of TRAIL, while human foreskin keratinocytes are not susceptible at those concentrations. The use of z-LEHD-FMK could completely protect the esophageal epithelial cells from TRAIL-mediated apoptosis without affecting cell death in TRAIL-sensitive esophageal cancer cells. These results using esophagus as a model are similar to what we previously reported[21] with human hepatocytes and other tumor types and thus, we believe that a brief period of caspase 9 inhibition during TRAIL administration may widen the therapeutic window and allow cancer cell killing while protecting normal cells. These results also suggest that different types of normal human tissues may display different sensitivities to TRAIL. We tested the effect of combining TRAIL and adriamycin on TRAIL-resistant esophageal cancer cell lines and on EPC2. Synergistic cell killing was observed both in TRAIL-resistant cancer cells and in EPC2. Then, we wondered whether the presence of z-LEHD-FMK could still protect EPC2 from apoptotic cell killing induced by the combination. We believe the results are exciting because

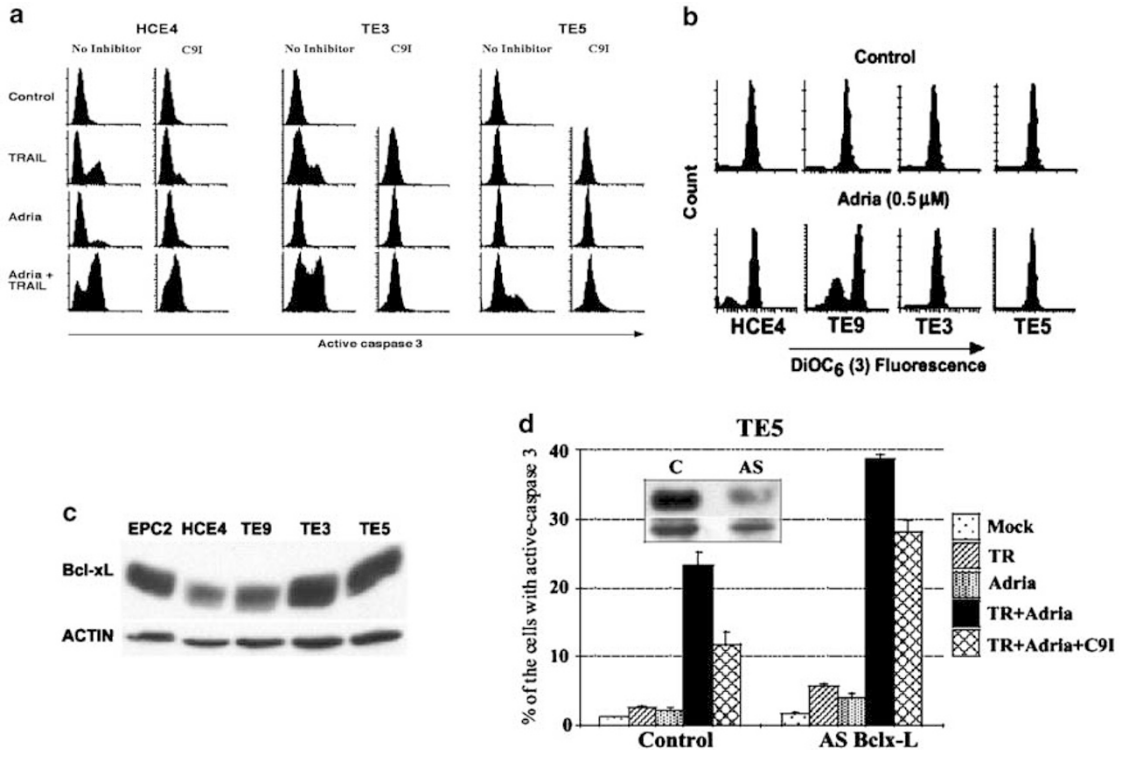

Figure 2 Targeting Bcl-x_L may result in sensitization of TRAIL-resistant esophageal squamous cell carcinomas to TRAIL, while use of caspase 9 blockade still permits death of the cancer cells by TRAIL plus adriamycin. (**a**) Active-caspase 3 assay for apoptosis after the combination of adriamycin and TRAIL treatment of TRAIL-resistant HCE4, TE3, and TE5 cells. The cells were pretreated with 0.5 μM adriamycin for 16 h followed by incubation for 4 h in the presence of TRAIL. z-LEHD-FMK (20 μM final concentration) was added (as indicated) at 2 h before the addition of TRAIL. (**b**) Mitochondrial membrane potential change after adriamycin treatment. To detect a change in the mitochondrial membrane potential ($\Delta\psi_m$) after treating cells with adriamycin, 3, 3′-dihexyloxacarbocyanine iodide ($DiOC_6$ (3)) was used. Briefly, 5×10^5 cells/well were seeded onto a six-well plate. After treatment with adriamycin (0.5 μM final concentration) for 16 h, the cells were harvested, washed and resuspended in complete media containing 100 nM $DiOC_6$ (3) followed by 30 min of incubation in a 37°C water-bath. After incubation, the change of $DiOC_6$ (3) fluorescence was analyzed by flow cytometry using a Beckman–Coulter Epics Elite analyzer. (**c**) Basal expression of Bcl-x_L in EPC2, HCE4, TE9, TE3, and TE5. To compare the expression level of Bcl-x_L, lysates were subjected to 15% SDS-PAGE. (**d**) Effect of suppression of endogenous Bcl-x_L in TE5. Either the morpholino-modified control oligonucleotide (Control or C in inset box) or the morpholino-modified antisense Bcl-x_L (AS Bcl-x_L or AS in inset) oligonucleotides were delivered to the cells by the scrape delivery method. The active-caspase 3 assay was performed after each treatment (as indicated). Insets show the expression level of Bcl-x_L (upper bands) and actin (lower bands) in TE5 cells after the corresponding oligonucleotide treatment. Morpholino-modified antisense Bcl-x_L oligonucleotides (5′-CGGTTGCTCTGAGA-CATTTTTATA-3′) designed to suppress expression of endogenous Bcl-x_L *in vivo*, and fluorescein-labeled standard control oligonucleotides were purchased from Gene Tools, LLC (Corvallis, OR, USA). The scrape delivery method was used to deliver the oligonucleotides to the cells. Briefly, cells (1×10^6/well) were seeded onto a six-well culture plate and after 16 h of incubation, the medium was changed to 1 ml of fresh medium containing 20 μM Morpholino-modified oligonucleotides. The cells were scraped out of the plates using a cell scraper (SARSTEDT, Newton, NC, USA). After two gentle up and down pipetting steps, the cells were transferred to a new culture plate. After 48 h of incubation, the cells were treated with the indicated conditions as described in the figure legends

the presence of z-LEHD-FMK could rescue EPC2 almost completely from cell killing by the combination. We found that the cancer cells (HCE4 and TE9) that were still killed even in the presence of z-LEHD-FMK showed a low basal expression of Bcl-x_L, and a drop of $\Delta\psi_m$ and Bax translocation after adriamycin treatment was observed only in those cells. The presence of the caspase 9 inhibitor did not affect the change in $\Delta\psi_m$ or bax translocation (data not shown). It has been suggested that antiapoptotic Bcl-2 family members, such as Bcl-2, and Bcl-x_L, may play central roles in preventing the loss of $\Delta\psi_m$, the release of cytochrome *c*, and the initiation of apoptosis,[20] and the amount of expression of endogenous Bcl-x_L inversely correlates with apoptotic cell death.[21] Although EPC2 apparently uses a type II pathway in TRAIL-mediated apoptotic signaling, TRAIL-sensitive esophageal cancer cells appeared to utilize a type I pathway. The present studies are beginning to unravel the molecular determinants as well as patterns of TRAIL sensitivity and resistance in esophageal squamous cell cancer. Bcl-x_L appears to be overexpressed in human squamous cell esophageal cancers and could contribute to TRAIL resistance. In cases where Bcl-x_L expression is high and contributes to TRAIL resistance, a strategy using antisense blockade of Bcl-x_L may resensitize the cells to TRAIL plus chemotherapy.

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