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ORIGINAL PAPER

Role of elevated pressure in TRAIL-induced apoptosis in human lung carcinoma cells

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Abstract TNF-related apoptosis-inducing ligand (TRAIL, Apo2L) is a promising anticancer agent with high specificity for cancer cells. Many strategies have been proposed to enhance the sensitivity of cancer cells to TRAIL-mediated apoptosis, including the use of combination treatment with conventional cancer therapies. However, few reports have evaluated the effects of TRAIL in combination with mechanical stress, which can also cause apoptosis of cancer cells. In the present study, we describe a custom-designed culture system that delivers two atmospheres of elevated pressure (EP) by using compressed air, and which enhances the sensitivity of cancer cells to TRAIL-mediated apoptosis. The combination of TRAIL and EP significantly increased apoptosis of human H460 lung cancer cells more than hyperbaric normoxia or normobaric mild hyperoxia. EP-potentiating TRAIL-mediated apoptosis of H460 cells was accompanied by up-regulated death receptor 5 (DR5), activation of caspases, decreased mitochondrial membrane potential, and reactive oxygen species production. We also observed EP-induced sensitization of TRAIL-mediated apoptosis in other cancer cell types. In contrast, human normal cells showed no DNA damage or cell death when exposed to the combined treatment. In a chicken chorioallantoic membrane model, EP enhanced TRAIL-mediated apoptosis of tumors that developed from transplanted H460 cells. Collectively, EP enhanced TRAIL-induced apoptosis of human lung carcinoma cells in vitro and in vivo. These findings suggest that EP is a mechanical and physiological stimulus that might have utility as a sensitizing tool for cancer therapy.

Keywords Elevated pressure · TNF-related apoptosis-inducing ligand (TRAIL) · Apoptosis · Cancer therapy

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Introduction

Recombinant TNF-related apoptosis-inducing ligand (TRAIL) is an attractive and promising anticancer agent that induces apoptosis of tumor cells [1, 2]. Unlike other members of the TNF superfamily (e.g., TNF and FasL), TRAIL targets cancer cells without causing harm to normal cells. The activity of TRAIL is dependent on the expression of cell membrane TRAIL receptors and caspase-8 [3], which initiates a protease cascade to activate effector caspases including caspase-3 and caspase-7 [4]. There are five receptors that can recognize TRAIL including DR4 and DR5, which have functional cytoplasmic death domains (DD). These death receptors trigger a TRAIL-induced apoptotic signal by forming a death inducing signal complex (DISC) that activates caspase-8 [5]. In the crosstalk between the extrinsic and intrinsic apoptosis pathways, mitochondrial signaling can amplify the signals initiated by the TRAIL receptors. Caspase inhibitors abrogate the majority of the cytotoxic effects of TRAIL,

reflecting the essential role of caspases in mediating the anticancer activity of TRAIL, as demonstrated in many experimental models [6–11]. Preclinical studies have shown that soluble forms of recombinant human TRAIL (rhTRAIL) suppress the growth of TRAIL-sensitive human tumor xenografts without systemic toxicity [12, 13]. This effect has also been observed in non-human primates [14]. Efforts to increase the sensitivity of tumor cells to TRAIL-mediated apoptosis include the use of a combination of different drugs with different mechanisms of action, such as those that activate distinct or overlapping apoptosis pathways. Natural or artificial compounds have been proposed to increase TRAIL sensitization by stimulating DR4 and DR5 activity, enhancing DISC formation, activating intracellular signal transduction, or down-regulating anti-apoptotic proteins [8, 15–17].

Recent strategies for designing cancer therapeutics have been aimed at the tumor microenvironment, which is made up of a complex network of tumor, endothelial, lymphatic, and fibroblast cells within an extracellular matrix (ECM). The growth and behavior of tumors is strongly influenced by characteristics of their microenvironment including hypoxia and vascularization. Reactive oxygen species (ROS) generation and oxygenation of the hypoxic tumor microenvironment are therefore strategies for cancer therapy. Treatment with ROS generators during chemotherapy and radiotherapy improves the response of many solid tumors [18–20]. However, the utility of ROS generators for cancer therapy is controversial because ROS also promote angiogenesis and tumor initiation [21–24].

Mechanical stresses are also important factors affecting the microenvironment of cells. Interstitial fluid flow in or around tissues influences the mechanical microenvironment. Pressure force, shear, or pulsative stress acts on the cell surface and ECM [25] to modulate tumor metastasis as well as anticancer drug delivery [26]. As a consequence of the application of extrinsic mechanical force to cells or tissues, the elevated pressure (EP) inhibits growth, as suggested by in vitro models of venous hypertension [27] and senescence [28–30]. However, there have been few reports evaluating the combined effects of TRAIL with mechanical stress, which could potentiate the apoptosis of cancer cells.

Our previous study described the development of a custom-designed cell culture system capable of synchronously regulating the pressure and oxygen levels within the culture microenvironment, which models the effects of mechanical/physiological stress on cells in vitro [28]. An elevated pressure of 2 ATA in this system induced stress-induced responses with mild hyperoxia, but without triggering severe oxidative stress [31]. In the present study, the effect of elevated pressure with mild oxygenation (EP, 2 ATA) was examined in combination with TRAIL therapy using NCI-H460 cells, which are a well characterized model system for the study of the mitochondrial apoptotic pathways and a representative non small cell lung carcinoma cell (NSCLC) line [32, 33]. Co-treatment with TRAIL and EP markedly sensitized NCI-H460 cells to TRAIL-induced apoptosis by activating the caspase-mediated apoptotic pathway. The TRAIL-sensitizing effect of EP was also shown in a chick chorioallantoic membrane model. The present study demonstrates the therapeutic potential of mechanical pressure stress for sensitizing tumors to TRAIL-based therapy.

Materials and methods

Cell lines and culture conditions

H460 (non-small cell lung carcinoma), WI-38 (normal fetal lung fibroblast), H1299 (non-small cell lung carcinoma), Jurkat (acute T cell leukemia), HepG2 (human hepatocellular carcinoma), CRT-MG (human glioblastoma), and HeLa (human epithelial cervical carcinoma) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), and were grown in RPMI 1640 medium or Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and antibiotics in an atmosphere of 95% air and 5% CO_2. Sixteen hours after plating, cultured cells were treated with various concentrations of TRAIL (Peprotech, Rocky Hill, NC), and then exposed to normoxia (air + 5% CO_2, $pCO_2 = 38$ mmHg, $pO_2 = 146.566$–147.288 mmHg) as a control condition, EP (elevated pressure with mild oxygenation, 2 ATA air + 5% CO_2, $pCO_2 = 38.228$ mmHg, $pO_2 = 305.9$–306.66 mmHg), or HN (hyperbaric normoxia, 2 ATA in normoxic conditions) for various times for each experiment.

In vitro cytotoxicity and apoptosis assay

Cell viability was measured using the MTT assay. The formazan reaction product was quantified spectrophotometrically at 570 nm using a VERSAmax ELISA reader (Molecular Devices, Sunnyvale, CA). Apoptosis was quantified using an Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Analyses were performed with a FACS Calibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

Measurement of dissolved oxygen and intracellular reactive oxygen species

The dissolved oxygen (DO) concentration of the media was measured using a Sension 6 Dissolved Oxygen Electrode

(HACH, Loveland, CO). Intracellular reactive oxygen species were measured by flow cytometry following staining with Dichlorofluorescein diacetate (DCFH-DA) obtained from Molecular Probes (Eugene, OR). Cells were incubated in serum-free medium with DCFH-DA (30 μM) at 37°C for 15 min. Intracellular ROS production, as determined by fluorescence intensity, was observed by fluorescence microscopy (Observer D1, Carl Zeiss, Düsseldorf, Germany).

Single-cell gel electrophoresis (SCGE, comet assay)

To measure DNA damage caused by EP and TRAIL in normal HDF, the alkaline comet assay was performed as previously described [28]. The slides were stained with Gel Red and examined using a Komet 5.5 image analysis system (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope in which the parameters of Olive tail moment (OTM) and tail distance (TD) were calculated automatically.

Flow cytometric analysis of TRAIL receptors

To quantify cell surface DR4 and DR5 levels, the suspended cells were directly incubated with PE-conjugated anti-DR4 or anti-DR5 antibodies at 4°C for 30 min. The cells were washed with PBS twice and resuspended in 0.5% BSA. Isotype IgG was used as the negative control. Analyses were performed with a FACS Calibur flow cytometer and CellQuest software (BD Biosciences).

Western blot analysis

Lysates (20 μg) were separated on 12.5% SDS gels before transfer to PVDF membranes (Millipore, Bradford, MA). The membranes were probed with antibodies against the proteins of interest, washed with Tween 20 in PBS, and incubated with peroxidase-conjugated secondary antibody. Immunoreactivities were detected using an ECL plus kit (GE Healthcare, Buckinghamshire, UK) and quantitative data were obtained using molecular imaging software (Kodak, New Haven, CT).

Mitochondrial outer membrane permeabilization assay

The mitochondrial membrane potential of cells was measured by using the Mitoprobe JC-Assay Kit (Molecular probe) with some modifications. Briefly, harvested cells were incubated in 1 μM JC-1 at 37°C and 5% CO_2 in a humidified incubator for 10–30 min. After two washes, the cells were resuspended in PBS and immediately analyzed on a FACS Calibur flow cytometer with Cell Quest software (BD Bioscience).

Caspase-8 activity assays

The specific proteolytic activity of caspase-8 was determined with a caspase-8 colorimetric assay kit (R&D system) following the manufacturer's instructions. The enzymatic reaction for caspase-8 activity was read at 405 nm with a microplate reader (Molecular Devices, Menlo Park, CA). As a negative control, Z-IETD-CHO (25 μM) was used to inhibit the activity of caspase-8.

Chick chorioallantoic membrane assay

The chorioallantoic membrane (CAM) assay was done as described previously [34, 35]. Windows of 2-cm diameter were opened on shells of fertilized eggs on day 9. H460 cells (2×10^6) were suspended in 50% Matrigel Matrix (BD Biosciences) and injected on the CAM of 10-day-old eggs. After developing a microtumor on the CAM, 3 ng of TRAIL in 15 μl of PBS was applied topically, once per day for 3 days.

Immunohistochemistry

Tumors from the CAM assay were sampled, fixed, paraffin embedded, and serially sectioned (5 μm). Immunohistochemical staining was performed with 1:1 hematoxiline, 0.5% eosin, and anti-ki67 (Dako, Carpinteria, CA). Apoptosis was assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL; Millipore) according to the manufacturer's instructions.

Statistical analysis

Data were expressed as the mean ± SE of three independent experiments. Student's *t* test and two-way ANOVA were used for statistical analysis and asterisks indicate statistical significance.

Results

Oxygen tension and intracellular ROS generation in response to elevated pressure

The effects of a mechanical stimulus on cell death were compared using four different conditions, including normal control (Ctr, $pO_2 = 146.566$–147.288 mmHg), elevated pressure with mild hyperoxia (EP, 2 ATA of the air, $pO_2 = 305.9$–306.66 mmHg), hyperbaric normoxia (HN, 2 ATA with 10% O_2, $pO_2 = 147$ mmHg), and normobaric mild hyperoxia (NMH, 1 ATA, 40% $O_2 = 304$ mmHg). To evaluate the level of physiological oxygen and the oxidative stress resulting from the various experimental

conditions, oxygen tension in the media and intracellular hydrogen peroxide were quantified. Dissolved oxygen increased 1.78-fold in EP conditions compared to control conditions (Fig. 1a). The dissolved oxygen in the EP conditions was similar to that in the NMH conditions, which did not trigger oxidative stress in normal lung fibroblasts for 48 h in a previous study [28]. The oxygen tension in the HN conditions was the same as in the control conditions. Intracellular ROS generation after 48 h in EP conditions was 1.5-fold greater than in control conditions (Fig. 1b). However, EP-induced apoptosis or DNA damage was not detected at significant levels by alkaline comet (Fig. 1c) or apoptosis assays (Fig. 1d). EP was not sufficient to trigger p53 activation. These results suggest that EP conditions act as a mild stimulator of ROS production by influencing the influx of air into H460 cells without triggering cell death or oxidative DNA damage in the short term.

Combined treatment of rhTRAIL with elevated pressure

To investigate the effects of EP, NMH, and HN on TRAIL-induced apoptosis, H460 cells were treated with rhTRAIL in the four conditions for 48 h and subjected to apoptosis assays. Cultures in all conditions showed a dose-dependent induction of apoptosis by TRAIL. Interestingly, potentiation of apoptosis was observed in cells treated with TRAIL and EP, with an approximate twofold increase in apoptotic events compared to other conditions (Fig. 2a). To confirm

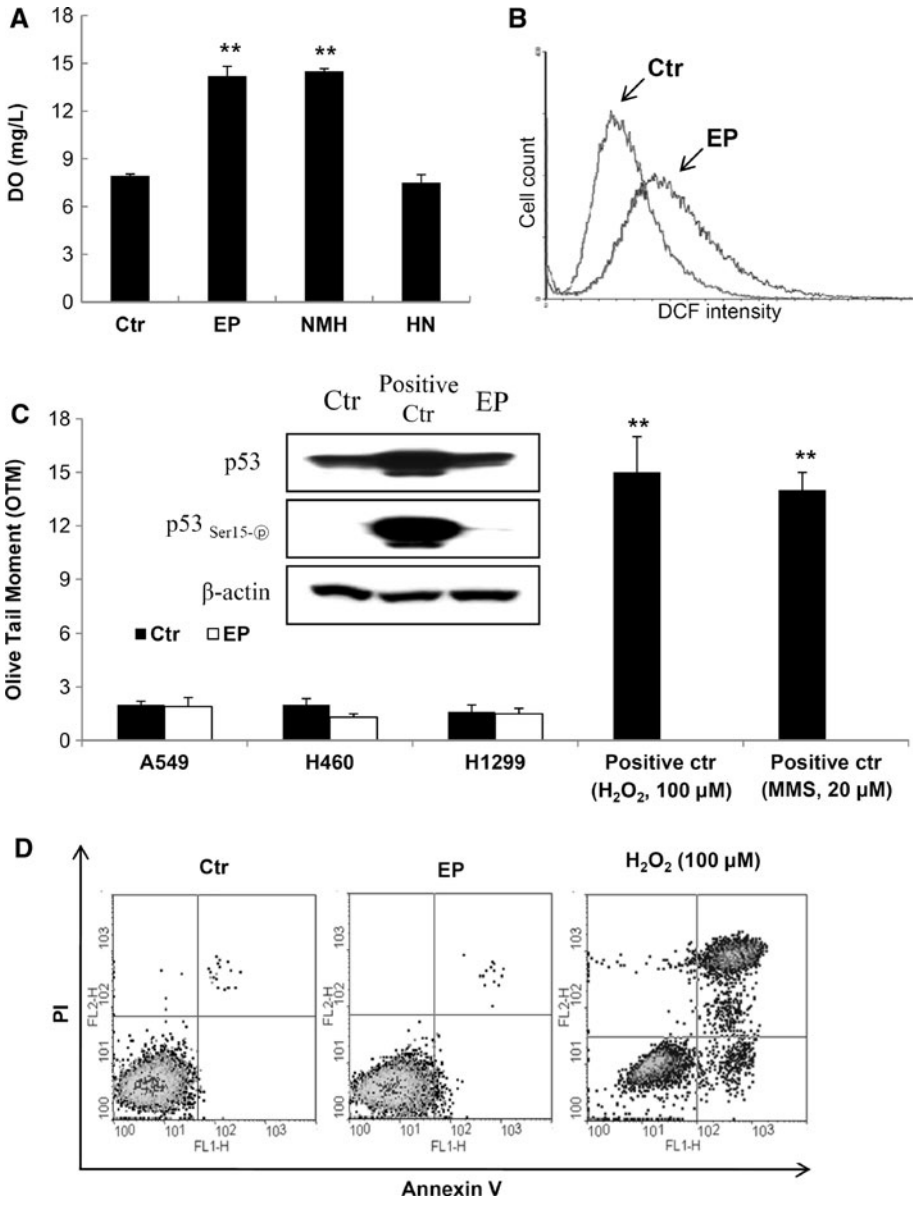

Fig. 1 EP elevates the oxygen tension of cell culture media and intracellular ROS levels without inducing cellular toxicity. **a** The dissolved oxygen (DO, unit of mg/ml) tension was analyzed in media exposed to EP, NMH, or HN for 6 h. When exposed to mild hyperoxic conditions (EP and NMH), DO increased and stabilized. **b** Intracellular ROS was measured by incubating cells with DCFH-DA, and DCF intensity was analyzed by flow cytometry. **c** Genotoxicity was determined by alkaline comet assay to detect DNA strand break damage. H460 cells treated with H_2O_2 or MMS were used as positive controls. Total or activated (serine phosphorylation) forms of p53 were analyzed by Western blotting. Cells were harvested after 2 days of elevated pressure (EP) exposure. H460 cells treated with Cisplatin (10 μg/ml) for 6 h were used for positive control for p53 activation. **d** AnnexinV and PI staining were performed at 48 h after initial exposure to EP. The mean difference was considered significant at ** $p < 0.0001$ compared to the control group

that the enhanced cell death was due to the TRAIL extrinsic death signal and not to a toxic side-effect of EP, TRAIL activity was blocked by pretreating with chimeric DR5-Fc. Inhibition of TRAIL activity completely rescued the EP-sensitized cell death (Fig. 2b). These results show that combined treatment of TRAIL with EP effectively sensitizes cancer cells to apoptosis mediated by the extrinsic pathway.

EP-mediated up-regulation of DR5 expression in H460 cells

To investigate the effect of EP on apoptosis pathways involved in TRAIL-mediated apoptotic signaling in human lung cancer cells, the expression of TRAIL receptors was evaluated by direct immunofluorescence staining with PE-conjugated antibodies and flow cytometry analysis of DR4 and DR5 expression at 24 h. Only DR5 expression was induced in H460 cells exposed to EP conditions (Fig. 2c, left). The relative changes of expression of death receptors and isotype IgG were expressed as ratios of the signal intensity in EP conditions over control conditions (Fig. 2c, right). The EP conditions selectively stimulated the expression of DR5, and this enhancement was associated with increased sensitivity to TRAIL-induced apoptosis.

Caspase activity in H460 cells treated with elevated pressure and rhTRAIL

To determine if EP and rhTRAIL combination treatment induces caspase activation, H460 cells were treated with TRAIL and/or EP for various time periods (2–6 h) and active forms of caspases were measured by Western blot analysis. As shown in Fig. 3a, procaspases-3,-8, and -9 were degraded faster in EP conditions than control conditions. In addition, the activated [cleaved form (CF)] caspases were more stable in H460 cells exposed to TRAIL and EP conditions than control conditions. The CFs of each caspase were quantified and expressed as fold changes (Fig. 3a, right). Cellular FLICE-inhibitory protein (c-FLIP)

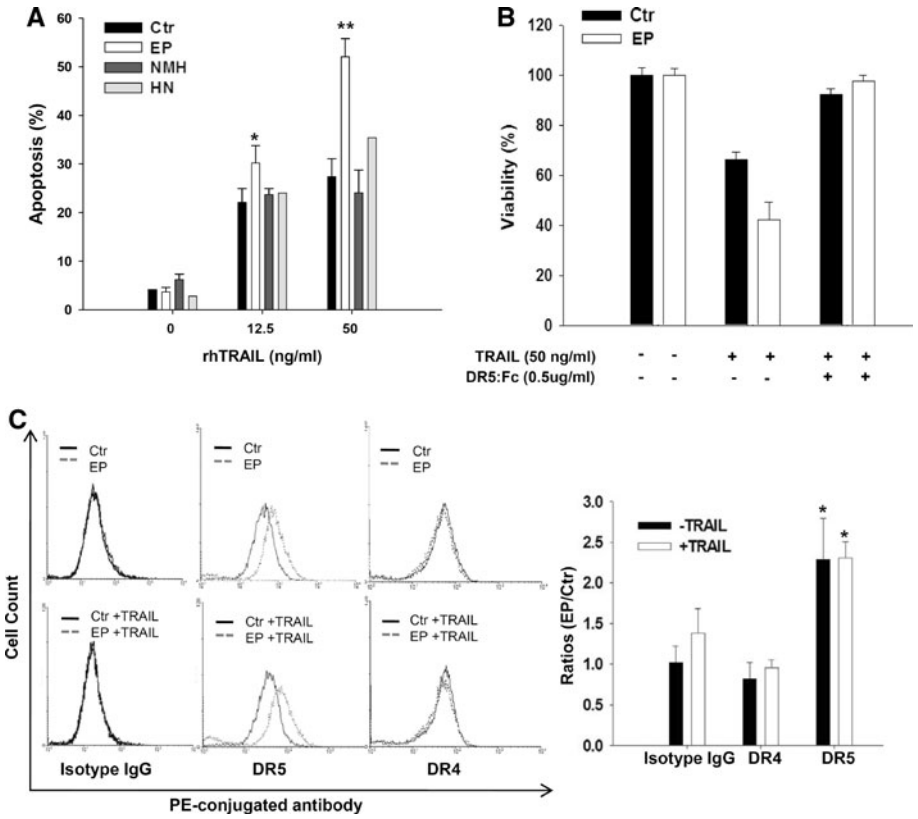

Fig. 2 EP sensitizes human NSCLC H460 cells to TRAIL-induced apoptosis. **a** Cells (24×10^4 cells per well) were subjected to the four conditions and treated with rhTRAIL (12.5 or 50 ng/ml) for 48 h. Cell death was measured with annexin-V and PI staining. **b** Cells were incubated with DR5:Fc chimeric protein (0.5 μg/ml) to block TRAIL activity for 2 h prior to TRAIL and EP treatment. Cell viability was determined by MTT assay at 24 h after treatment with TRAIL and/or EP. **c** Expression of death receptors (DR4 and DR5) on H460 cells grown in each condition (±TRAIL 50 ng/ml) was determined by flow cytometry. Cells treated with EP alone or EP and TRAIL were harvested and incubated with PE-conjugated antibodies. The relative changes in the expression levels of death receptors and isotype IgG were expressed as the ratios of the signal intensity in EP conditions over the signal in control conditions. Significant differences (* $p < 0.05$ and ** $p < 0.001$) compared to the control group

is a primary inhibitor of TRAIL-mediated apoptosis, which inhibits caspase-8 activation by preventing recruitment of capase-8 to DISCs [36]. The levels of c-FLIP (both long and short) are regulated by ubiquitin/proteasome-mediated degradation [37, 38]. c-FLIP$_L$ was degraded faster in cells exposed to EP and TRAIL than TRAIL alone. That means that EP efficiently induces TRAIL-DISC formation and caspase-8 activation, triggering the apoptotic pathway [39]. Western blot assays showed that IAPs such as XIAP and survivin were down-regulated, and cleavage of PARP 1/2 was significantly higher in H460 cells treated with TRAIL and EP than control conditions (Fig. 3b). The enhanced activation of caspase-8 cleavage was also detected by the colorimetric caspase-8 assay in vitro. A dose dependent increase in caspase-8 activity was observed in H460 cells treated with TRAIL and EP, and the increase in caspase-8 activity was completely abrogated by Ac-IETD-CHO, a caspase-8 inhibitor (Fig. 3c). Treatment with caspase

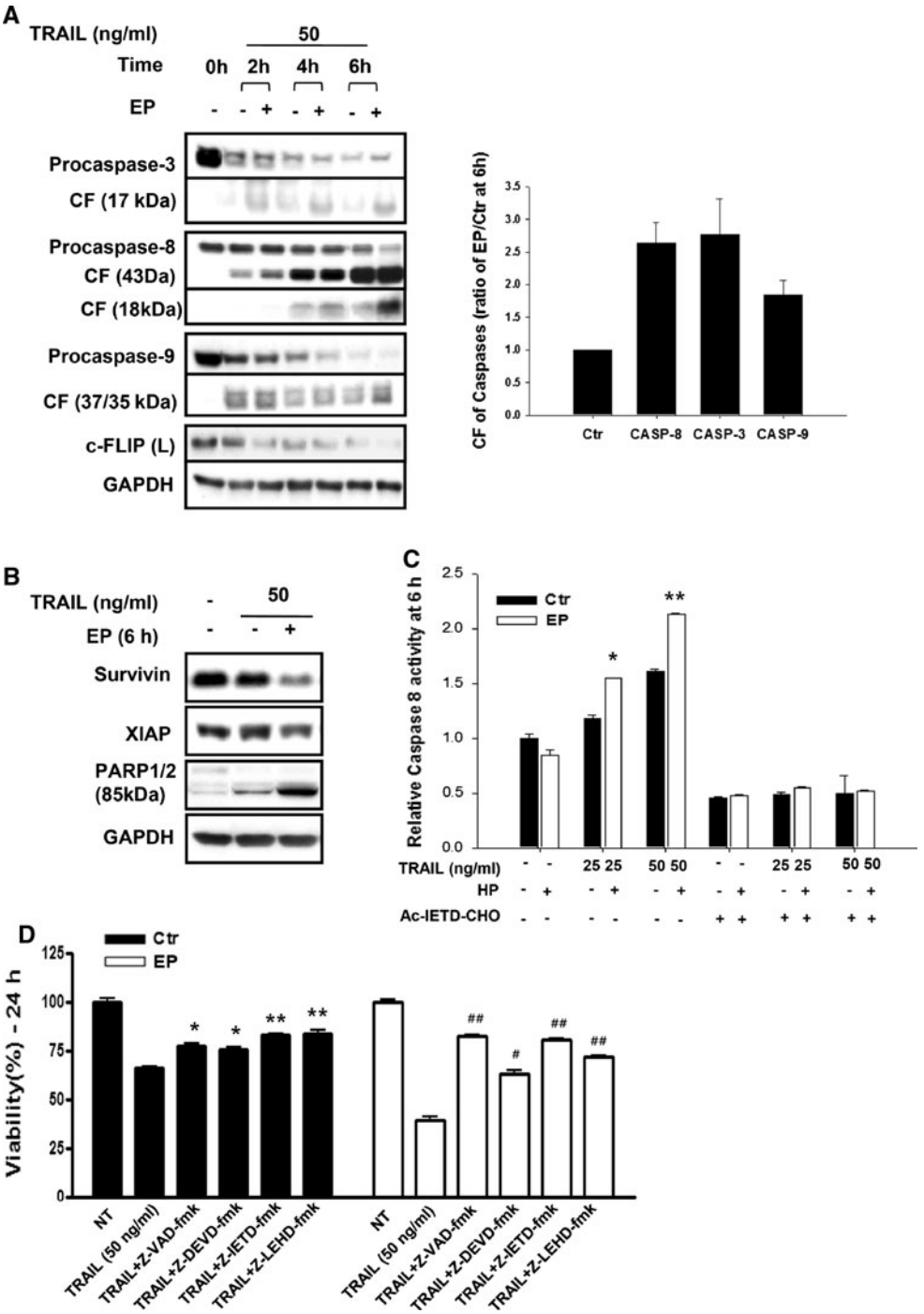

Fig. 3 Treatment with rhTRAIL/EP activates caspase-3, -8, and -9 in human NSCLC H460 cells. After treatment with rhTRAIL (50 ng/ml) and EP, the levels of the cleaved forms (CFs) of caspases, c-FILP (**a**), Survivin, XIAP, and PARP1/2 (**b**), were detected at various times (2, 4, or 6 h) by Western blot analysis. CFs of caspases at 6 h are shown. **c** Caspase-8 activity was determined with a colorimetric caspase-8 assay kit. A reversible inhibitor of caspase-8 (Ac-IETD-CHO), was used to measure non-specific hydrolysis of the substrate Ac-IETD-AMC. Caspase-8 activity was expressed as the fold change relative to untreated cells in control conditions. **d** Cell viability was determined by the MTT assay at 24 h after treatment with general caspase, caspase-3, caspase-8, or caspase-9 inhibitors (irreversible and cell permeable inhibitors, 10 μM of Z-VAD-fmk, Z-DEVD-fmk, Z-IETD-fmk, and Z-LEHD-fmk, respectively). *(#) $p < 0.001$ and **(##) $p < 0.0001$ comparing viability of cells treated with TRAIL and/or EP to those with inhibitors

inhibitors also rescued cell viability. These results indicated that the sensitized cell death was induced by a caspase dependent mechanism (Fig. 3d).

Activation of the intrinsic pathway by Bid cleavage and the mitochondrial outer membrane permeabilization

Because EP stimulation coupled with TRAIL treatment increased caspase-9 activation, several mitochondrial pathway pro-apoptotic or anti-apoptotic molecules were investigated in cells exposed to EP (Fig. 4a). Bid cleavage, a mediator of the extrinsic death signal, was dramatically activated and bcl-2 was slightly degraded by TRAIL and EP co-treatment. The concentrations of Bax and cytochrome *c* released into the cytosol were higher after TRAIL and EP co-treatment than TRAIL alone. To assay mitochondrial depolarization, JC-1 staining was performed in H460 cells undergoing apoptosis after TRAIL treatment alone or TRAIL with EP co-treatment. Co-treatment with EP increased mitochondrial outer membrane permeabilization (MOMP), which sensitized cells to TRAIL-mediated apoptosis (Fig. 4b). DCF intensities were observed under a fluorescence microscope to measure intracellular ROS generation during TRAIL-induced apoptosis. An increase in ROS production was associated with enhanced cell death in response to co-treatment with TRAIL and EP. This result indicates that the EP conditions activated MOMP (Fig. 4c). However, MOMP and ROS levels were not significantly different in cells exposed to EP alone when compared to control conditions at 6 h. These results strongly suggested that stimulation with EP directly activated the TRAIL-

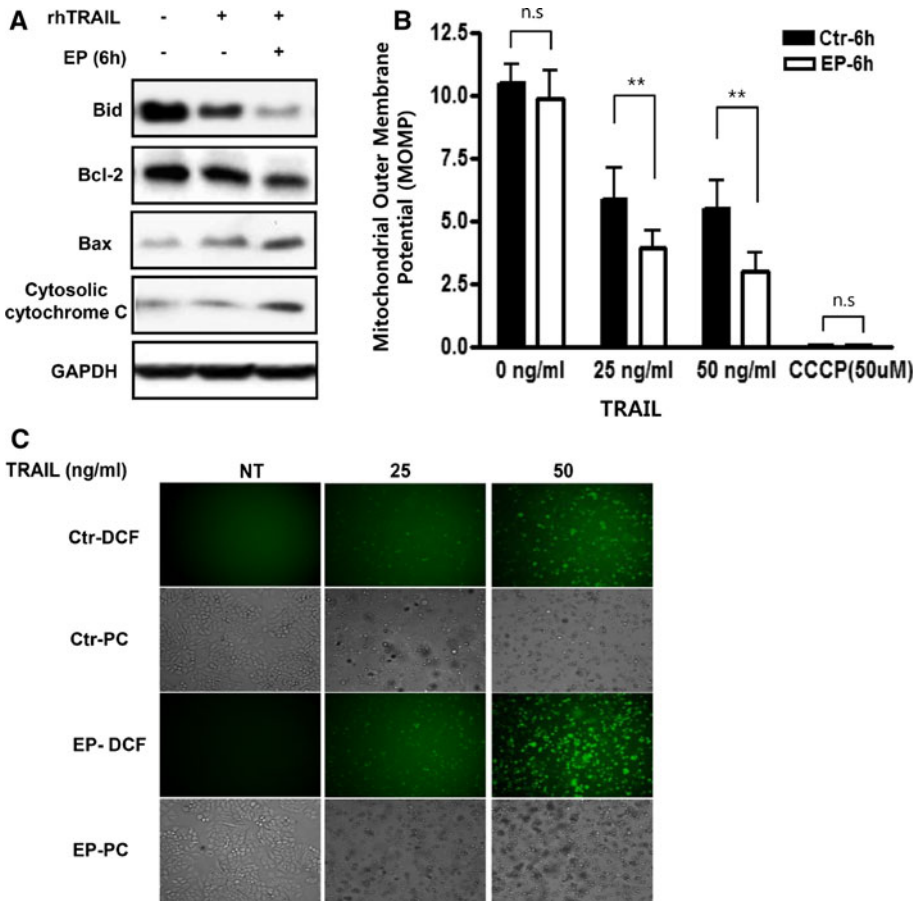

Fig. 4 The mitochondrial apoptotic pathway is activated in H460 cells during TRAIL-induced apoptosis in response to EP. **a** Protein lysates derived from H460 cells were probed for Bid, Bcl-2, Bax, and GAPDH by Western blotting. Cytosolic cytochrome *c* was isolated from lysates in hypotonic buffer by ultracentrifugation. Protein levels were determined by Western blotting after the purity of the cytosolic fraction was tested for deficiency of COX IV. **b** To determine MOMP at 24 h, cells treated with rhTRAIL alone or rhTRAIL and EP were incubated with JC-1, a mitochondrial specific fluorescent dye, and analyzed by FACS. MOMP was expressed as the ratio of monomers (FL1) to aggregates (FL2). CCCP, a mitochondrial membrane-potential disrupter, was used as a positive control. **c** During apoptosis at 6 h, intracellular ROS levels were evaluated using fluorescence microscopy to detect intracellular dichlorofluorescein (DCF) fluorescence. Cell images were acquired by using the phase-contrast mode (PC). Fluorescence in cells is shown in green. Significant difference of ** $p < 0.001$ compared to the control group

mediated mitochondrial apoptotic pathway in human lung cancer cells.

Combined treatment with TRAIL- and EP-enhanced apoptosis of several cancer cell lines but not primary HDF

The effect of TRAIL and EP co-treatment was evaluated in various human cancer cell lines. Most cancer cells tested showed increased TRAIL-mediated apoptosis when exposed to EP for 48 h (Fig. 5a). Of the cell lines tested, CRT-MG and HeLa cells were particularly sensitive to EP stress, showing approximately twofold more TRAIL-induced apoptosis in response to TRAIL and EP co-treatment than with TRAIL alone. For the A549 cell line, a highly resistant cell line, apoptosis was slightly enhanced, but was insufficient to overcome their resistance to TRAIL (Fig. 5b). Yet EP dramatically enhanced apoptosis of CRT-MG, a human astroglioma cell line. Thus, it seems that EP can efficiently facilitate TRAIL-mediated apoptosis but not overcome the resistance of all cancer cell lines. It shows that sensitizing effect of EP is variable to cell type dependent manner. This combined treatment did not trigger apoptosis (Fig. 5c) or genotoxicity (Fig. 5d) of human normal fibroblasts (WI-38).

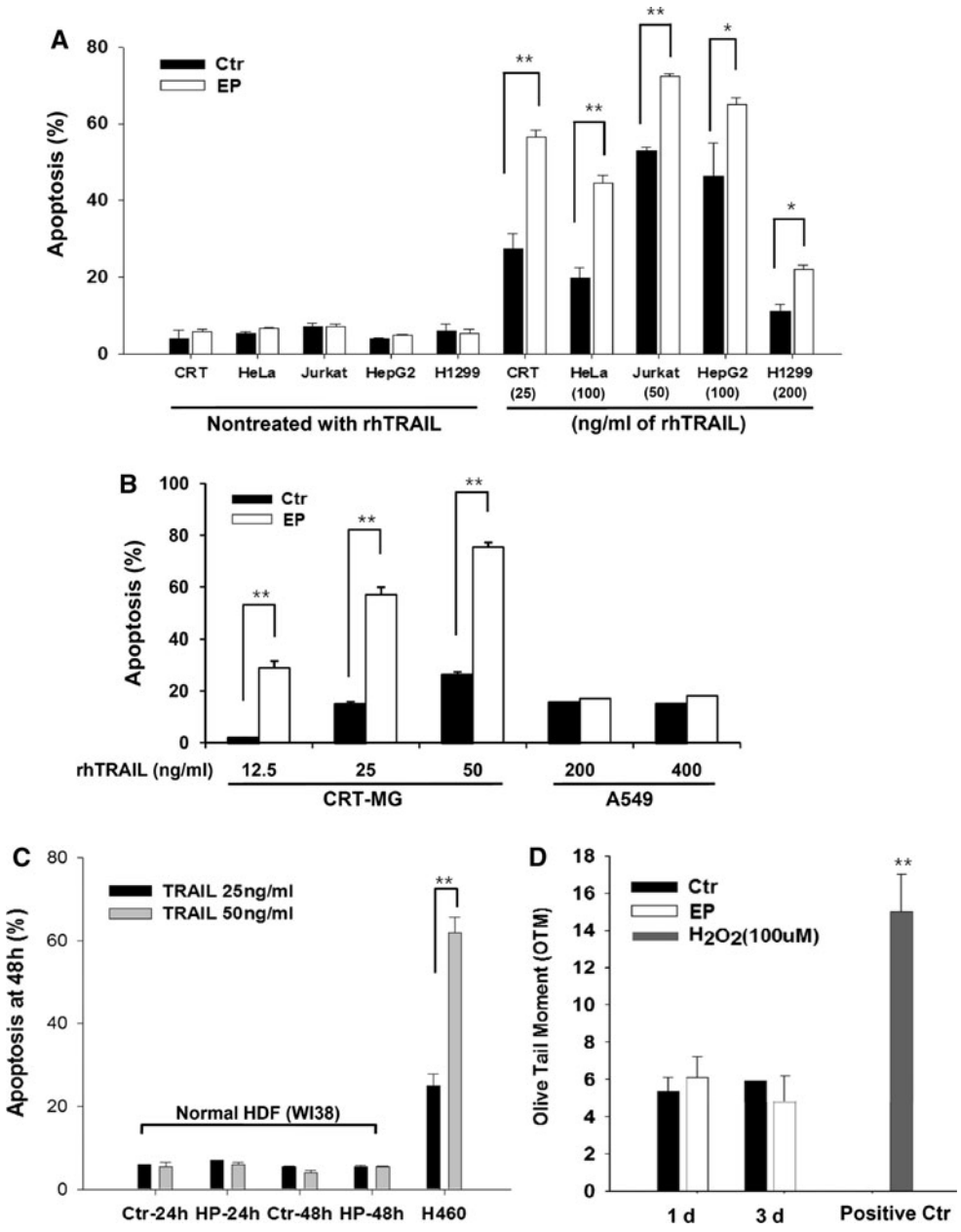

Fig. 5 Various human cancer cell lines showing sensitization to TRAIL-induced apoptosis after EP treatment, without harmful effects to normal fibroblasts. **a** H460, WI-38, H1299, Jurkat, HepG2, CRT-MG, and HeLa cells were tested for EP-enhancement of TRAIL effects at 48 h. EP alone did not trigger apoptosis of cancer cells (*left*). *Numbers* in parenthesis refer to the concentrations of rhTRAIL were strongly potentiated by EP (*right*). Cell death was measured with annexin V and PI. **b** Apoptosis in CRT-MG and A549 was determined by annexin V/PI staining assay. Two cell lines were treated with combined treatment (EP) or TRAIL alone (Ctr) for 2 days. **c** Cytotoxicity and genotoxicity of normal lung fibroblasts (WI-38) were tested to assess the safety of EP stress. Cytotoxicity of rhTRAIL (25 and 50 ng/ml) and EP on normal WI-38 cells was determined by annexinV and PI staining. H460 cells were used as positive control for TRAIL-mediated apoptosis. **d** After exposure to EP for 1 or 3 days, the comet assay was performed to evaluate DNA damage in WI-38 cells. Cells bearing DNA damage (positive cells) were prepared by treating cells with H_2O_2 (100 μM). Significant differences of * $p < 0.05$ and ** $p < 0.001$ compared to the control group, respectively

Enhanced TRAIL-induced antitumor activity against NSCLC in vivo

To evaluate the antitumor activity of combined treatment with TRAIL and EP in vivo, we used an established in vivo xenograft tumor model system known as the CAM model [34]. To develop the tumor mass, H460 cells were transplanted on the CAM of chick embryos, allowed to form tumors for 4 days, and then treated with a combination of PBS, TRAIL, and EP for 3 days. EP conditions alone were non-toxic, as shown by the viability and normal growth (body weight) of the embryos (Fig. 6a). Combined treatment with TRAIL and EP had a stronger apoptotic effect on NSCLC microtumors than TRAIL-only or mock-treated controls, as visualized in tissue sections (Fig. 6b–d). Compared to TRAIL-only exposed embryos, the population of proliferative cells that were positively stained with Ki67 was significantly decreased in EP/TRAIL co-treated embryos (Fig. 6c). Conversely, the population of apoptotic cells positively stained by the TUNEL assay was significantly higher (Fig. 6d). This result suggests that EP stimulation cooperates with TRAIL in vivo to activate the apoptosis pathway and inhibit growth of NSCLC cells. These findings show that EP enhances TRAIL-mediated antitumor activity in vivo, resulting in the suppression of tumor growth without detectable toxicity to embryos, thereby suggesting that EP might have utility as a sensitizing tool for cancer therapy.

Discussion

We have demonstrated that the combined use of TRAIL and EP enhances the apoptosis of cancer cells by triggering apoptotic pathways without causing harmful side effects to normal cells. The mechanical stress induced by EP sensitized cells and tumors to TRAIL-mediated apoptotic signaling. Mechanical (physical) stresses including microenvironmental factors such as shear, friction, tension, and viscosity have recently been reported to act as modulators of stem-cell differentiation [40, 41], apoptosis [42], and senescence [43]. Researchers have introduced various methods to generate mechanical stress that can be used to

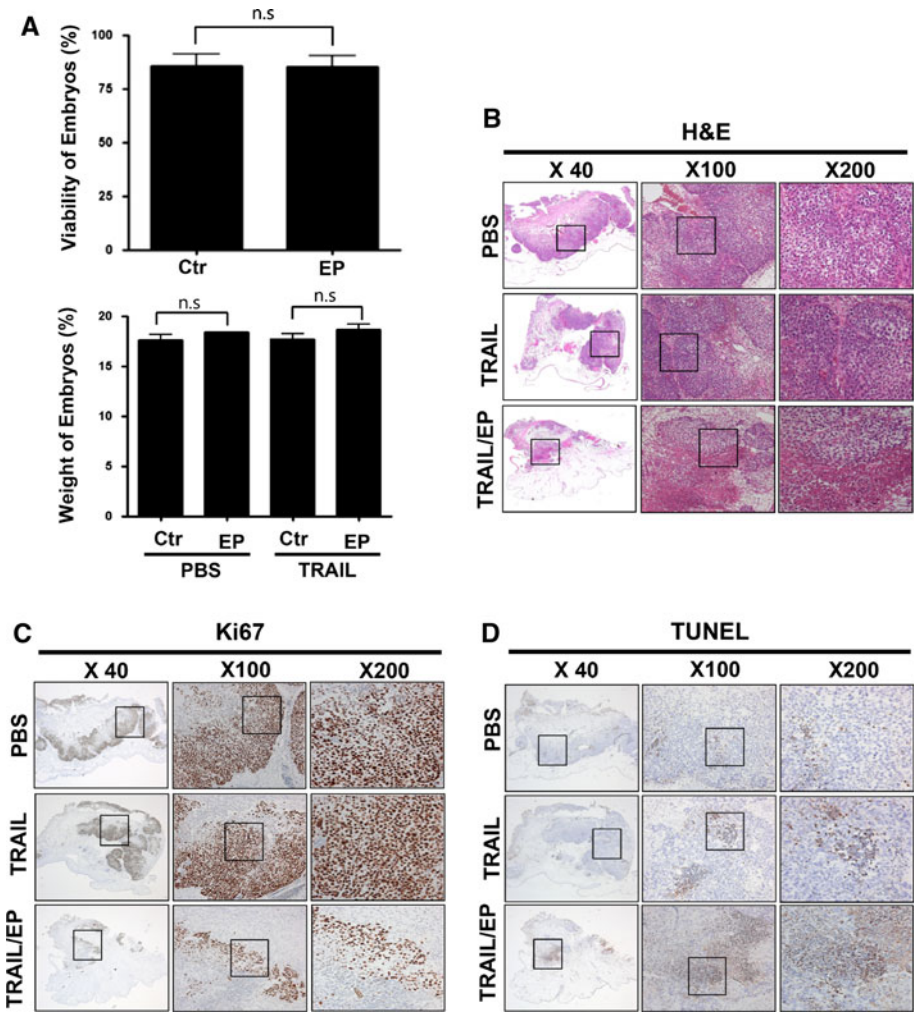

Fig. 6 EP enhances TRAIL-induced antitumor activity against NSCLC tumors in vivo. The CAM microtumors were treated for 3 days with 3 ng of TRAIL and/or EP. PBS treatment was used as a mock-control. To evaluate the status of embryo development, viability of embryos was checked daily and each embryo was weighed after final treatment (**a**). The CAM tissue was excised, fixed, and stained with H&E, anti-Ki67, and TUNEL. Representative images are shown in **a** (H&E), **b** (Ki67), and **c** (TUNEL)

target phenomena in particular cell types including mesenchymal stem cells, cancer cells, and primary cultured cells. Meanwhile, hyperoxygenation (HBO) has been shown to sensitize tumors to conventional cancer therapy by increasing the intratumoral oxygen tension and ROS production through the influx of pure oxygen with high pressure [18]. Although many reports have indicated that HBO is beneficial for the treatment of cancer because it sensitizes cells to chemotherapy and radiotherapy, there have also been reports of harmful side effects including oxidative DNA damage and gene/chromosome mutations caused by oxygen toxicity [24, 44]. Combination therapies with HBO have focused on elevating intratumoral oxygen tension without considering the effect of elevated pressure.

We investigated the use of mechanical stress without oxygen toxicity to sensitize cancer therapy. Our in vivo model system studies suggest that the combination of TRAIL and EP has potential utility as a novel cancer therapy. Although the molecular mechanisms behind the observed induction of cancer cell death by EP-sensitized TRAIL treatment remain to be clarified, we have shown that EP increases apoptosis of lung cancer cells without harmful effects in vitro and in vivo. In this study, the generation of ROS alone did not explain the TRAIL-EP enhancement of apoptosis because the cells exposed to TRAIL-NMH did not show a significant increase in cell death compared to cells exposed to TRAIL alone. Although the intracellular ROS level was increased under EP conditions in the present study, EP-specific enhancement of TRAIL-mediated apoptosis was not accompanied by ROS-mediated p53 activation (Fig. 1c). ROS generated by EP was not sufficient to trigger DNA damage or apoptosis (Figs. 1c, 4b). The increase in ROS production may have been related to high oxygen partial pressure (Fig. 1a, b). Increased generation of ROS in response to TRAIL and EP co-treatment may have been caused by the disruption of the mitochondrial outer membrane during apoptosis of cancer cells. While the TRAIL-EP-induced generation of ROS directly stimulated the intrinsic apoptosis pathway in H460 cells, pressure without mild hyperoxia (HN) did not potentiate TRAIL-induced apoptosis. We propose that mechanical pressure stress with elevated oxygen tension stimulated TRAIL-induced apoptosis, through the generation of mild ROS. The biological interface between mechanical and physiological stresses, such as ROS, should be explored in future studies.

Regarding the relationship between TRAIL and ROS, several reports describe sensitization to TRAIL-induced apoptosis through a mechanism involving ROS generation. The increased intracellular ROS generated by MG132 or hydrogen peroxide sensitizes cells to TRAIL-induced apoptosis of astrocytic cancer cells [45] and NSCLCs [16] by activating NF-κB and its possible target gene, death receptor 5. The expression of death receptors plays an important role in the sensitization to TRAIL-induced apoptosis. In the present study, EP-specific up-regulation of DR5, but not DR4, was observed in H460 cells. The expression of TRAIL receptors (DR4 and DR5) is regulated by a newly identified p53-responsive intronic element. The activation of p53 by ROS leads to the up-regulation of DR5 gene expression and sensitization to TRAIL [16]. However, in our study pretreatment with antioxidants such as *N*-acetylcysteine (NAC), PBN (phenyl-α-tert-butyl nitrone), and catalase failed to block EP-induced DR5 up-regulation and apoptosis, suggesting that ROS are not required for EP-induced DR5 expression in the combined treatment conditions (data not shown). Interestingly, c-FLIP, bcl-2, Survivin, and XIAP, which are well characterized inhibitors of apoptosis that confer TRAIL resistance to cancer cells, were down-regulated in our study. Many reports have suggested that silencing or knockout of IAP family proteins may enable anticancer drugs to activate the intrinsic apoptotic pathway [46, 47].

Our studies support the potential utility of EP as a co-treatment to sensitize cancer cells to TRAIL-mediated anti-tumor therapy. However, the intracellular sensors that trigger apoptosis by up-regulating DR5 and down-regulating anti-apoptotic protein levels remain elusive. Furthermore, the variable sensitizing effect of EP on different cancer cell types will provide more sensitizing mechanisms in different background of cancer cells against anti-cancer drug effect.

In addition, defining the effects of EP on ECM and the mechanosensitive nature of mechanical stress is a critical research goal because accumulating evidence points to the importance of the biophysical properties of the ECM and other structural proteins as possible mediators of the molecular mechanism is known to modulates the response of tumors and their phenotypes to mechanical stress [48]. Mechanosensory transduction systems are responsible for the critical regulation of microenvironmental changes [49, 50]. Given that TRAIL is currently in clinical trials to assess anticancer efficacy and safety, our combination therapy approach with EP has promising therapeutic utility for the TRAIL therapy.

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