Hydrogen peroxide induces cell death in human TRAIL-resistant melanoma through intracellular superoxide generation

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Abstract. Intracellular reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are thought to mediate apoptosis induced by death receptor ligands, including tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). However, the role of H_2O_2 is controversial, since some evidence suggests that H_2O_2 acts as an anti-apoptotic factor. Here, we show that exogenously applied H_2O_2 (30-100 μM) induces cell death in TRAIL-resistant human melanoma cells via intracellular superoxide (O_2^-) generation. H_2O_2 induced apoptotic or necrotic cell death, depending on the concentration of the oxidant applied; low concentrations of H_2O_2 preferentially activated the caspase-dependent apoptotic pathway, while high concentrations of H_2O_2 induced apoptotic and necrotic cell death in a caspase-independent manner. The H_2O_2-induced cell death was associated with increased mitochondrial membrane potential collapse and caspase-3/7 activation and ER stress responses including caspase-12 and X-box-binding protein-1 (XBP-1) activation. H_2O_2 induced intracellular O_2^- generation even within the mitochondria, while TRAIL did not. The superoxide dismutase mimetic antioxidant MnTBaP [Mn (III) tetrakis (4-benzonic acid) porphyrin chloride] inhibited the H_2O_2-induced O_2^- generation, apoptosis and XBP-1 and caspase-12 activation at comparable concentrations. Importantly, H_2O_2 treatment caused minimal O_2^- generation and apoptosis in normal primary melanocytes. These data show that H_2O_2 induces endoplasmic reticulum-associated cell death via intracellular O_2^- generation and that malignant melanoma cells are more susceptible than normal cells to this oxidative cell death. The findings suggest that H_2O_2 has therapeutic potential in the treatment of TRAIL-resistant melanoma.

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Introduction

Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor cytokine superfamily and has a homotrimeric structure. Since TRAIL induces apoptosis in a variety of transformed and cancer cells, but not in normal cells, it is a promising target for cancer prevention and treatment. However, growing evidence suggests that some cancer cell types such as malignant melanoma, glioma and non-small cell lung cancer (NSCLC) cells are resistant to TRAIL-induced apoptosis (1). Moreover, TRAIL-responsive tumors acquire a resistant phenotype that renders TRAIL therapy ineffective. Overcoming the TRAIL resistance of cancer cells is necessary for effective TRAIL therapy and drugs potentiating TRAIL effectiveness are urgently required (2). TRAIL binds to receptors (DRs) that contain death domains such as death receptor (DR) 4/TRAIL-receptor 1 (TRAIL-R1) and DR5/TRAIL-R2 (3). This binding induces oligomerization of the receptors and conformational changes in the death domains, resulting in the formation of a death-inducing signaling complex; and subsequent activation of caspase-8. In turn, activated caspase-8 activates the effector caspase-3/6/7, which executes the apoptotic process (4,5). The activation of caspase-8 is also linked to the intrinsic (mitochondrial) apoptotic pathway. Activated caspase-8 can cleave and activate the pro-apoptotic Bcl-2-family molecule Bid, which in turn activates other Bcl-2-family molecules, Bax and Bak, resulting in their oligomerization and the formation of megachannels in the outer mitochondrial membrane. The release of cytochrome *c* through the Bax/Bak megachannels into the cytosol induces the assembly of apoptosome and the activation of caspase-9, resulting in the activation of caspase-3/6/7 (4). Recently, various pro-apoptotic receptor agonists such as recombinant human TRAIL and agonistic antibodies against DR4/DR5 have been subjected to clinical trials in a variety of cancer cell types, including malignant melanoma and NSCLC cells. Unfortunately, the results showed that these receptor agonists were disappointedly only modestly effective (6). Induction of apoptosis by the intrinsic pathway is considered to be the major mechanism of conventional chemotherapy and is therefore a critical target in cancer treatment. However, clinical observations suggest that amplification of the known apoptotic pathways is not sufficient for overcoming TRAIL resistance in cancer cells.

Reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) are products of normal metabolism in virtually all aerobic organisms and are also produced following xenobiotic exposure. Low physiological levels of ROS function as second messengers in intracellular signaling and are required for normal cell function, while excessive ROS cause damage to multiple macromolecules, impair cell function, and promote cell death (7). Imbalance between ROS production and the ability of a biological system to detoxify oxidants or to repair the resulting damage leads to oxidative stress. Several lines of evidence suggest that intracellular H_2O_2 is a mediator of DR ligand-induced apoptosis in tumor cells. The flavonoid wogonin kills TNF-α-resistant T-cell leukemia cells and sensitizes them to TNF-α- or TRAIL-induced apoptosis by increasing intracellular H_2O_2 levels (8). LY303511 sensitizes human neuroblastoma cells to TRAIL, and intracellular H_2O_2 generation plays a role in this effect (9). On the other hand, some evidence suggests that H_2O_2 acts as an anti-apoptotic factor in DR ligand-induced apoptosis. The continuous presence of low concentrations of H_2O_2 inhibits caspase-mediated apoptosis in Jurkat cells by inactivating pro-caspase-9 (10). It was reported that in human astrocytoma cells, H_2O_2 is generated in a caspase-dependent manner and contributes to resistance to TRAIL-induced apoptosis (11). Thus, the role of H_2O_2 in DR ligand-induced apoptosis is controversial.

Here we demonstrate that H_2O_2 induces cell death in TRAIL-resistant melanoma cells via intracellular O_2^- generation. Our data suggest that the intracellular O_2^- mediates ER-associated cell death in these cells. Importantly, normal primary melanocytes were much lesser sensitive than malignant melanoma cells to oxidative cell death.

Materials and methods

Chemicals and antibodies. Reagents were obtained from the following manufacturers: soluble recombinant human TRAIL, Enzo Life Sciences (San Diego, CA); DATS, Wako Pure Chemicals (Osaka, Japan); thapsigargin, Sigma-Aldrich (St. Louis, MO); z-VAD-fluoromethylketone (fmk) (VAD), z-DEVD-fmk (DEVD), z-IETD-fmk (IETD), z-LETD-fmk (LETD) and Mn (III) tetrakis (4-benzonic acid) porphyrin chloride (MnTBaP), Calbiochem (La Jolla, CA). z-LEVD-fmk (LEVD) and z-ATAD-fmk (ATAD), BioVision (Mountain View, CA); dichlorohydrofluorescein diacetate (DCFH-DA), dihydroethidium (DHE), MitoSOX™ Red (MitoSOX) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benz-imidazolocarbocyamine iodide (JC-1), Life Technologies Japan (Tokyo, Japan). Reagents were dissolved in dimethylsulfoxide and diluted with Hanks' balanced salt solution (HBSS; pH 7.4) to a final concentration of <0.1% before use. Dimethylsulfoxide alone at a concentration of 0.1% (vehicle) showed no effects throughout this study. Polyclonal antibodies against X-box-binding protein-1 (XBP-1) and glucose-related protein 78 (GRP78) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All the other chemicals were of analytical grade.

Cell culture. Human A2058 and SK-MEL-2 melanoma cells were obtained from Health Science Research Resource Bank (Osaka, Japan). Human A375 melanoma cells were obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in high glucose-containing Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) in a 5% CO_2-containing atmosphere. The cells were harvested by incubation in 0.25% trypsin-EDTA medium (Life Technologies Japan) for 5 min at 37˚C. Normal human epidermal melanocytes were obtained from Cascade Biologics (Portland, OR) and cultured in DermaLife Basal medium (Kurabo, Osaka, Japan) supplemented with DermaLife M LifeFactors (Kurabo) in a 5% CO_2-containing atmosphere. The cells were harvested by incubation in 0.25% trypsin-EDTA medium for 5 min at 37˚C.

Determination of cell death by fluorescence microscopy. The overall cell death was evaluated by performing fluorescence microscopy as previously described (12). Briefly, the cells ($1x10^4$ cells) were placed on 8-chamber coverslips (Asahi Glass Co., Tokyo, Japan) and treated with the agents to be tested for 24 h at 37˚C in a 5% CO_2-containing atmosphere. The cells were then stained with 4 μM each of calcein-AM and ethidium bromide homodimer-1 (EthD-1) to label live and dead cells, respectively, using a commercially available kit (LIVE/DEAD® Viability/Cytotoxicity kit; Life Technologies Japan) according to the manufacturer's instructions. Images were obtained using a fluorescence microscope (IX71 inverted microscope, Olympus, Tokyo, Japan) and analyzed using the LuminaVision software (Mitani Corporation, Fukui, Japan).

Determination of apoptotic cell death. Apoptotic cell death was quantitatively assessed as previously described (12). Briefly, the cells plated in 24-well plates ($2x10^5$ cells/well) were treated with TRAIL and the agents to be tested alone or together for 20 h in DMEM containing 10% FBS (FBS/DMEM). Subsequently, the cells were stained with FITC-conjugated Annexin V and PI using a commercially available kit (Annexin V FITC Apoptosis Detection Kit I; BD Biosciences, San Jose, CA). The stained cells were analyzed in a FACSCalibur flow cytometer (BD Biosciences) using the CellQuest software (BD Biosciences). Four cellular subpopulations were evaluated: viable cells (Annexin V^-/PI^-); early apoptotic cells (Annexin V^+/PI^-); late apoptotic cells (Annexin V^+/PI^+); and necrotic/damaged cells (Annexin V^-/PI^+). Annexin V^+ cells were considered to be apoptotic cells.

Measurements of mitochondrial membrane potential ($\Delta\Psi_m$) depolarization and caspase-3/7 activation. $\Delta\Psi_m$ depolarization and caspase-3/7 activation were simultaneously measured by a previously described method (12). Briefly, the cells plated in 24-well plates ($2x10^5$ cells/well) were treated with the agents to be tested in FBS/DMEM for 24 h, stained with the dual sensor MitoCasp (Cell Technology Inc., Mountain View, CA), and analyzed for their caspase-3/7 activity and $\Delta\Psi_m$ in the FACSCalibur using the CellQuest software. Changes in $\Delta\Psi_m$ were also measured using the lipophilic cation JC-1 by a previously described method (13).

Measurement of caspase-12 activation. Activated caspase-12 in living cells was detected using the caspase-12 inhibitor ATAD conjugated to FITC as previously described (12). This compound binds to active caspase-12, but not to inactive caspase-12. The

cells ($2x10^5$ cells/ml) were stained with FITC-ATAD for 30 min at 37˚C using a CaspGLOW Fluorescein Caspase-12 Staining Kit (BioVision) according to the manufacturer's protocol. Fluorescence was determined using the FL-1 channel of the FACSCalibur and analyzed using the CellQuest software.

Measurement of intracellular ROS. The production of intracellular ROS was measured using the oxidation sensitive DHE and DCFH-DA by flow cytometry by a previously described method (14). Briefly, cells ($4x10^5$/500 μl) resuspended in HBSS were treated with the agents to be tested and incubated at 37˚C for various time periods, then incubated with 5 μM DHE or DCFH-DA for 15 min at 37˚C. The cells were washed, resuspended in HBSS on ice and centrifuged at 4˚C. The green fluorescence (DCFH-DA) and red fluorescence (DHE) were measured using the FL-1 and FL-2 channels of the FACSCalibur, respectively, and analyzed using the CellQuest software. Mitochondrial O_2^- generation was measured using the mitochondria-targeting probe MitoSOX Red as previously described (14). Briefly, the cells ($4x10^5$/500 μl) suspended in HBSS were treated with the agents to be tested and incubated at 37˚C for various time periods, then incubated with 5 μM MitoSOX Red for 15 min at 37˚C. The cells were washed, resuspended in HBSS on ice, and centrifuged at 4˚C. The red fluorescence was measured using the FL-2 channels of the FACSCalibur and analyzed by CellQuest software.

Detection of intercellular ROS by fluorescent microscopy. The cells ($1x10^4$) were plated on 8-chamber cover glasses and treated with the agents to be tested for 30 min at 37˚C in a 5% CO_2 containing atmosphere. After removal of the medium, the cells were stained with 4 μM each of DCFH-DA and DHE to label cells producing H_2O_2 and O_2^-, respectively. Images were obtained with the fluorescence microscope and analyzed using LuminaVision software.

Western blot analysis. Western blot analysis was carried out by the previously described method (12). The cells in 6-well plates ($1x10^6$ cells/ml/well) were treated with the agents to be tested for 24 h at 37˚C, washed and lysed with SDS-sample buffer. The whole cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes (Nippon Millipore, Tokyo, Japan). After blocking the membranes with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) at room temperature for 60 min, GRP78 and XBP-1 proteins on the membranes were detected using specific antibodies. Antibody-antigen complexes were detected using the ECL Prime Western Blotting Reagent (GE Healthcare Japan, Tokyo, Japan). To verify equal loading, the membranes were re-probed with an antibody against β-actin or GAPDH.

Results

H_2O_2 induces cell death in human TRAIL-resistant melanoma cells. First, we examined the effect of exogenously applied H_2O_2 on melanoma cell survival. After treatment with H_2O_2 at varying concentrations, A375 cells were stained with calcein-AM and EthD-1 and subjected to fluorescence microscopic analysis. Live cells were stained green with calcein-AM, while dead cells with compromised cell membranes were stained red with EthD-1. As shown in Fig. 1A, treatment with 100 μM H_2O_2 for 24 h resulted in considerable cell death, while treatment with 100 ng/ml TRAIL had a marginal effect. Similarly, A2058 cells and SK-MEL-2 cells were killed by H_2O_2, but not by TRAIL (Fig. 1B and C). In addition, more pronounced cell death was observed in the cells treated with TRAIL and H_2O_2 than in the cells treated with either of the agents alone. The cytotoxic effects of H_2O_2 on TRAIL-resistant melanoma cells were confirmed by apoptosis measurements using Annexin V/PI staining. H_2O_2 treatment resulted in a dose- and time-dependent increase in apoptosis in the A375 cells (Fig. 1D and E). Treatment with H_2O_2 up to 30 μM for 24 h resulted in only a modest (maximum 15%) increase in apoptosis (Annexin V^+ cells) and a moderate (35%) apoptosis was observed after 72 h. Treatment with 100 μM H_2O_2 for 24 h caused a moderate apoptosis and 70-90% of the cells underwent apoptosis at 72 h. While 30 μM H_2O_2 primarily caused apoptosis with minimal induction of necrosis, 100 μM H_2O_2 appeared to also cause necrosis, since not only Annexin V^+ but also Annexin V^-/PI^+ cells were increased. The effect varied considerably in different experiments depending on the basal level of Annexin V^-/PI^+ cells. The level varied ranging from 1.6-9.2% and when the level was relatively high, necrotic cells were increased up to 25%. These data suggest that under certain circumstances, these cells spontaneously undergo necrosis and that high concentrations of H_2O_2 promotes this process. Consistent with the analysis by fluorescent microscopy, TRAIL alone caused minimal apoptosis for 72 h. However, higher amplitude of apoptosis was observed in the cells treated with TRAIL plus 30 μM H_2O_2, but not TRAIL plus 100 μM H_2O_2 (Fig. 1F). H_2O_2 induced apoptosis in a dose-dependent manner in all the cell lines tested while substantial amplification of TRAIL-induced apoptosis by H_2O_2 was observed in some but not all cell lines (data not shown). The amplification was more pronounced with H_2O_2 at low concentrations than with H_2O_2 at high concentrations. These data show that H_2O_2 treatment induces cell death in human TRAIL-resistant melanoma cells. Subsequently, we investigated the H_2O_2-induced cell death in greater detail using A375 cells as a model cell system.

H_2O_2 induces melanoma cell death in a caspase-dependent or -independent manner, depending on the concentration applied. To understand the mechanisms underlying the H_2O_2-induced cell death, we examined whether or not the cell death was caspase-dependent. Analysis using caspase-specific inhibitors revealed that H_2O_2-induced apoptosis exhibited discriminate sensitivities to the given caspase inhibitor depending on the concentration of the oxidant applied. The general caspase inhibitor VAD pronouncedly inhibited the apoptosis induced by 30 μM H_2O_2 (maximum of 63% inhibition), while the caspase-3/7 inhibitor DEVD reduced it by 40% (Fig. 2A). Strikingly, the caspase-12 inhibitor ATAD was as effective as VAD and was significantly more effective than DEVD at inhibiting apoptosis. By contrast, the caspase-4 inhibitor LEVD enhanced rather than suppressed apoptosis. Interestingly, all these inhibitors minimally inhibited the apoptosis induced by 100 μM H_2O_2 (Fig. 2B). We further examined the effects of H_2O_2 on the $\Delta\Psi_m$ and caspase-3/7 activation, as these are hallmarks of the intrinsic apoptotic

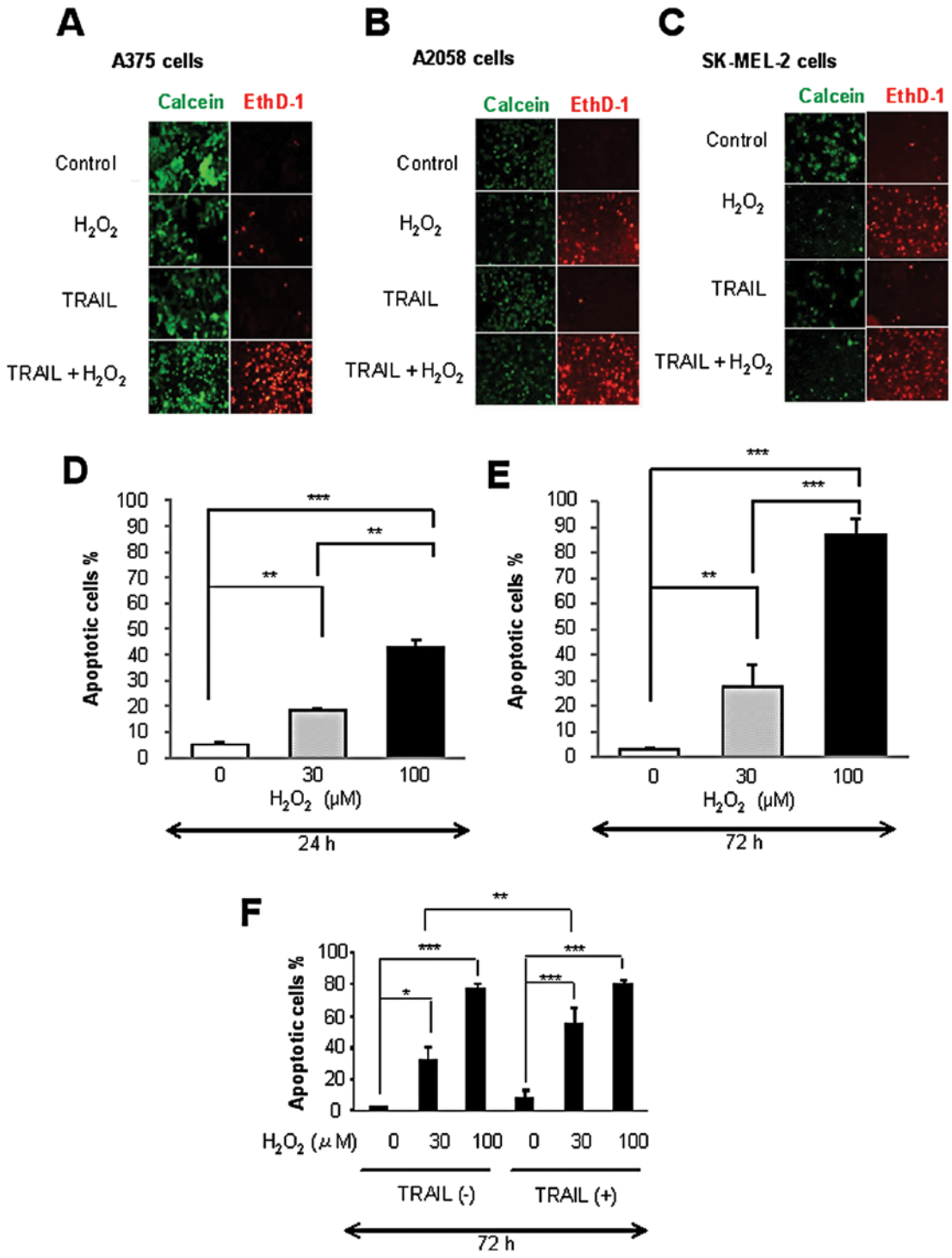

Figure 1. H_2O_2 induces cell death in TRAIL-resistant human melanoma cells. (A) Human A375, (B) A2058 and (C) SK-MEL-2 melanoma cells were treated with 30 μM H_2O_2 and 100 ng/ml TRAIL alone or in combination for 24 h. After removal of the medium, the cells were stained with calcein-AM and ethidium bromide homodimer-1 (EthD-1) to label live cells (green) and dead cells with compromised cell membranes (red), respectively. Images were obtained using a fluorescence microscope (x100). The results shown are representative of 4 independent experiments. (D-F) A375 cells were treated with 30 or 100 μM H_2O_2 and 100 ng/ml TRAIL alone or in combination for (D) 24 or (E,F) 72 h, stained with FITC-Annexin V/PI and analyzed by flow cytometry. Annexin V^+ cells were considered to be apoptotic cells. The data represent means ± SE from 4 to 8 independent experiments. $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

pathway. Flow cytometric analysis using the fluorescent $\Delta\Psi_m$-sensitive dye or the caspase-3/7-specific probe revealed that H_2O_2 induced $\Delta\Psi_m$ depolarization and caspase-3/7 activation in a dose-dependent manner (Fig. 2C and D). These data show that H_2O_2 stimulates multiple death pathways including intrinsic apoptotic and caspase-independent apoptotic and necrotic death pathways, depending on the concentration applied.

H_2O_2 induces intracellular O_2^- generation, which mediates apoptosis in human TRAIL-resistant melanoma cells. To elucidate the potential role of ROS in the H_2O_2-induced cell death,

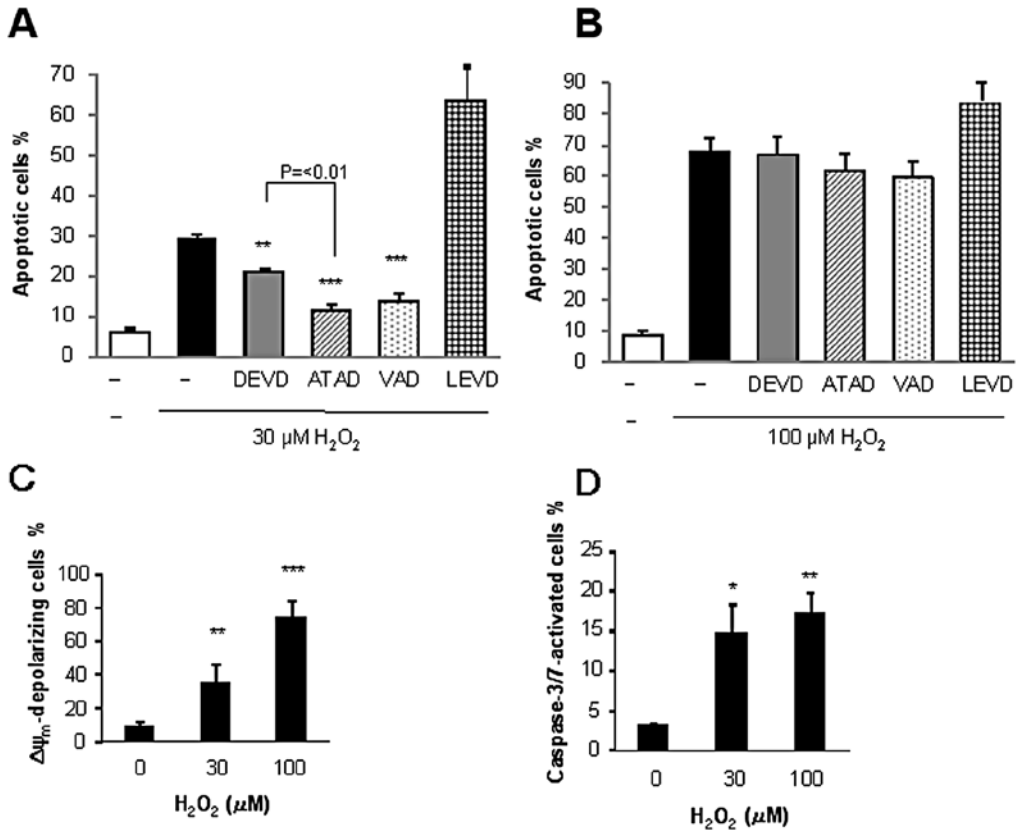

Figure 2. H_2O_2 induces melanoma cell apoptosis in a caspase-dependent or -independent manner, depending on the concentration applied. A375 cells were treated with (A) 30 or (B) 100 μM H_2O_2 for 24 h, in the presence or absence of 30 μM each of z-DEVD-fmk (DEVD, specific for caspase-3/7), z-ATAD-fmk (ATAD, specific for caspase-12), z-LEVD-fmk (LEVD, specific for caspase-4) and z-VAD-fmk (VAD, general caspase inhibitor) for 24 h and apoptotic cells were measured by performing Annexin V/PI staining. The data represent means ± SE from 5 to 7 independent experiments. **p<0.01; ***p<0.001. (C and D) A375 cells were treated with 30 or 100 μM H_2O_2 for 24 h. Loss of (C) mitochondrial membrane potential ($\Delta\Psi$m) and (D) caspase-3/7 activation were measured using the dual sensor MitoCasp by flow cytometry. The data represent means ± SE from 4 independent experiments. *p<0.05; **p<0.01; ***p<0.001.

we analyzed the generation of intracellular ROS after H_2O_2 treatment using the oxidation-sensitive dye DCF or DHE. DCF can react with multiple oxidants such as H_2O_2, peroxynitrite ($ONOO^-$) and •OH, but an increase in DCF fluorescence can be considered to primarily represent increased H_2O_2 level, since H_2O_2 is the most stable among these DCF-reacting oxidants. On the other hand, DHE undergoes two-electron-oxidation to form DNA-binding ethidium bromide; the reaction is mediated by O_2^-, but not by H_2O_2 or $ONOO^-$. Consequently, DCFH-DA and DHE have been widely used to assess the generation of intracellular H_2O_2 and O_2^-, respectively, in various rodent and human non-transformed and transformed cells (15-18). A375 cells were treated with H_2O_2 for 30 min and analyzed for their DCF or DHE fluorescence by performing fluorescence microscopy. As shown in Fig. 3A, only a modest increase in DCF (green) fluorescence, but not DHE (red) fluorescence, was observed in TRAIL-treated cells. This increase in DCF fluorescence was transient and declined to below the basal level within 1 h. On the other hand, unexpectedly, H_2O_2 treatment increased not only DCF fluorescence but also DHE fluorescence, indicating the production of intracellular O_2^-. This oxidative response was confirmed by flow cytometric analysis. The increase in the DHE signal was initially observed at 1 h (Fig. 3B), and it lasted for at least 4 h; the signal was completely abolished by superoxide dismutase (SOD) mimetic antioxidant MnTBaP (Fig. 3C).

Because mitochondria are the major site of ROS generation under physiological conditions, we examined the role of this organelle in H_2O_2-induced O_2^- generation. MitoSOX localizes to the mitochondria and serves as a fluoroprobe for selective detection of O_2^- in the organelle (15,19). As shown in Fig. 3D, a substantial increase in the MitoSOX signal was initially observed at 1 h after treatment and the increase lasted for at least 4 h; again, MnTBaP completely abolished the effect (Fig. 3E). Similar effects were also observed in A2058 cells (data not shown). To understand the role of intracellular O_2^- in H_2O_2-induced cell death, we examined the effect of MnTBaP treatment on cell death. The antioxidant blocked apoptosis in a dose-dependent manner (Fig. 3F). In addition to apoptosis, necrotic cell death induced by 100 μM H_2O_2 was also reduced. On the contrary, catalase had no effect on the cell death (data not shown). These results show that H_2O_2, but not TRAIL, induces the generation of intracellular O_2^-, including inside the mitochondria in TRAIL-resistant melanoma cells and that the O_2^- mediates apoptosis.

H_2O_2 induces ER stress responses while scavenging of O_2^- inhibits them. Caspase-12 is ubiquitously expressed and is localized to the ER membrane. It is specifically activated by ER stress to play a key role in the stress-induced apoptosis (22-25). Consequently, the data obtained suggested the

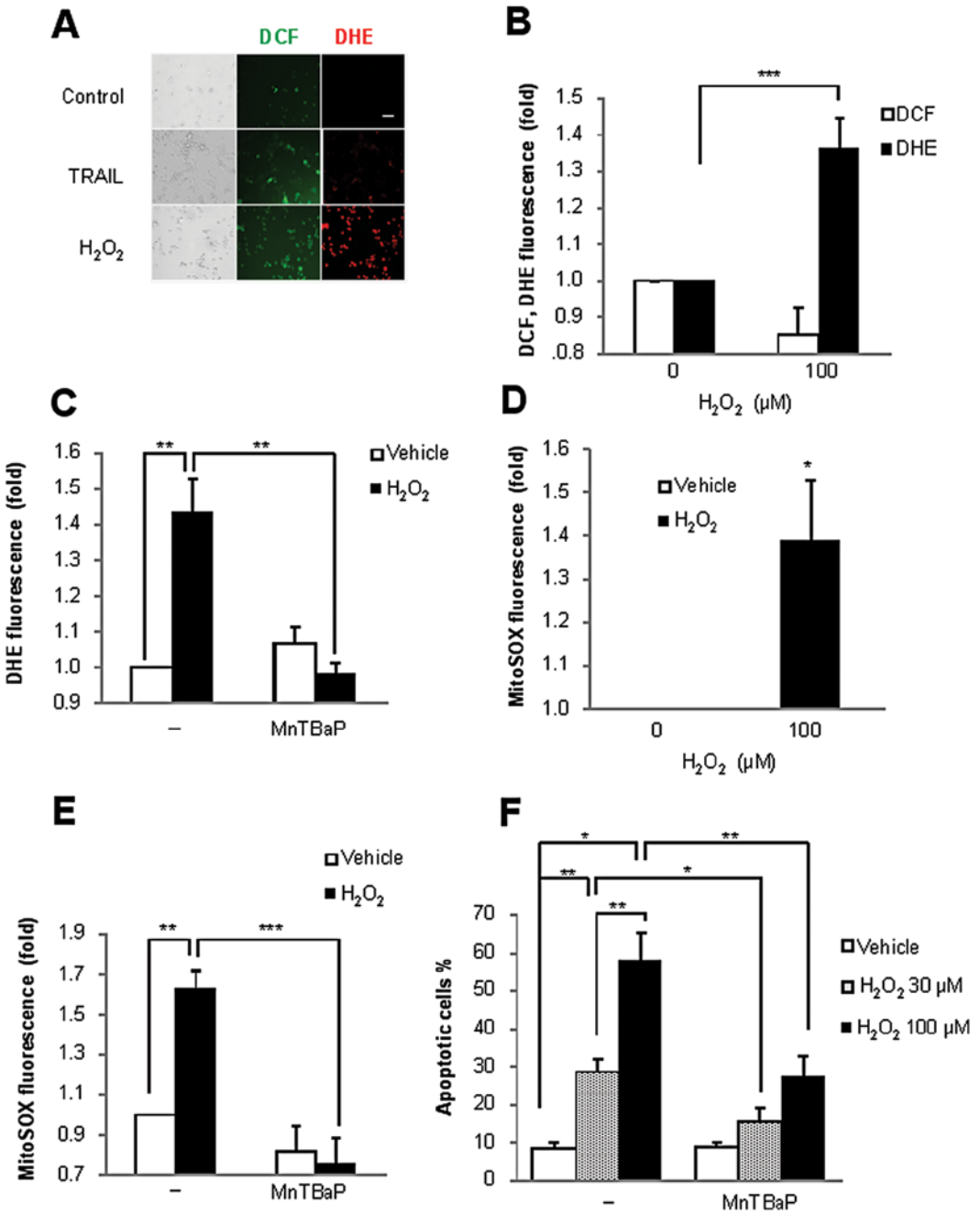

Figure 3. H_2O_2 induces intracellular O_2^- generation, which mediates apoptosis in human TRAIL-resistant melanoma cells. (A) A375 cells were treated with 100 μM H_2O_2 or 100 ng/ml TRAIL for 30 min. After removal of the medium, the cells were stained DCFH-DA (green) and DHE (red) to detect intracellular H_2O_2 and O_2^-, respectively. Images were obtained with a fluorescence microscope (x100; scale bar, 100 μm). The results shown are representative of 3 independent experiments. (B) A375 cells were treated with 100 μM H_2O_2 for 60 min and intracellular ROS production was measured using DHE and DCFH-DA by flow cytometry. The data represent means ± SE from 3 independent experiments. ***p<0.001. (C) A375 cells were treated with 100 μM H_2O_2 for 4 h in the presence or absence of 30 μM MnTBaP and intracellular O_2^- generation was measured by flow cytometry. The data represent means ± SE from 7 independent experiments. **p<0.01. (D) A375 cells were treated with 100 μM H_2O_2 for 60 min and mitochondrial O_2^- production was measured using the mitochondria-targeting O_2^- probe MitoSOX Red by flow cytometry. The data represent means ± SE from 7 independent experiments. *p<0.05. (E) A375 cells were treated with 100 μM H_2O_2 for 4 h in the presence or absence of 30 μM MnTBaP and mitochondrial O_2^- generation was measured by flow cytometry. The data represent means ± SE from 6 independent experiments. **p<0.01; ***p<0.001. (F) A375 cells were treated with 30 or 100 μM H_2O_2 for 24 h in the presence or absence of 30 μM MnTBaP, stained with Annexin V/PI and analyzed for apoptotic cell death by flow cytometry. The data represent means ± SE from 5 independent experiments. *p<0.05; **p<0.01.

possible role of ER stress including caspase-12 activation in oxidative cell death. To test this view, we examined whether H_2O_2 modulates caspase-12 activation. Fluorometric analysis using FITC-ATAD revealed that H_2O_2 induced caspase-12 activation in a dose-dependent manner at concentrations that effectively induced apoptosis (Fig. 4A and B). Furthermore, MnTBaP treatment blocked H_2O_2-induced caspase-12 activation. Treatment with MnTBaP (100 μM) almost completely abolished the effect of 30 μM H_2O_2 and reduced the effect of 100 μM H_2O_2 by 50% (Fig. 4C). These data show that scavenging of O_2^- inhibits H_2O_2-induced cell death and caspase-12 activation.

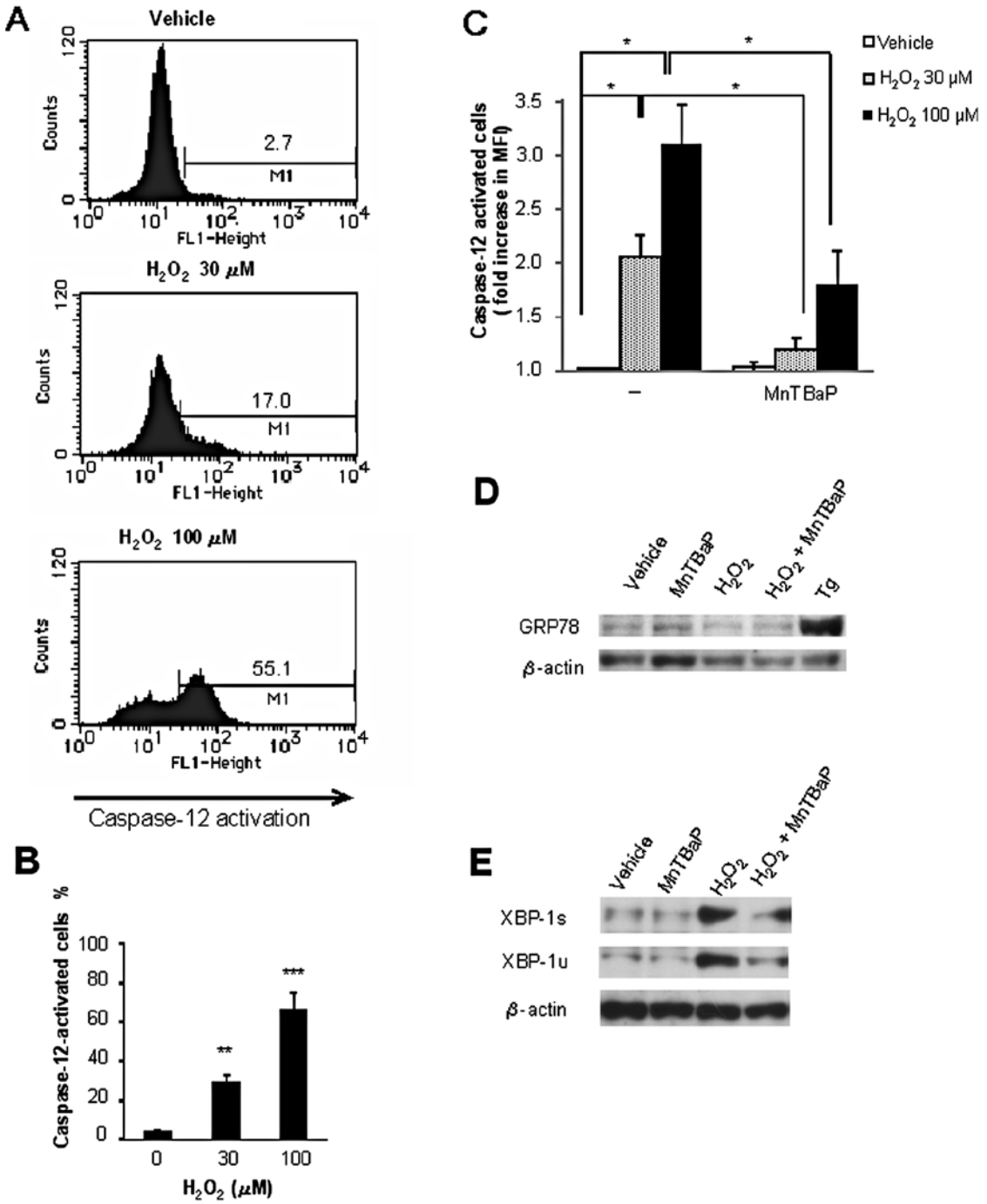

Figure 4. H_2O_2 induces ER stress responses and scavenging of O_2^- inhibits them. (A and B) A375 cells were treated with 30 or 100 μM H_2O_2 for 24 h and functional activation of caspase-12 was assessed by measuring the conversion of a cell-permeable substrate, FITC-ATAD-fmk by flow cytometry. Panel A shows typical histogram. The data shown in panel B represent means ± SE from 4 independent experiments. **p<0.01; ***p<0.001. (C) A375 cells were treated with 30 or 100 μM H_2O_2 for 24 h in the presence or absence of 30 μM MnTBaP and caspase-12 activation was assessed by flow cytometry. The data represent means ± SE from 4 independent experiments. *p<0.05. (D and E) A375 cells were treated with 100 μM H_2O_2 and 30 μM MnTBaP alone or in combination. Thapsigargin (Tg, 1 μM) served as a positive control of ER stress response. The cells were then washed, lysed with SDS-sample buffer and analyzed for GRP78 and XBP-1 content by western blot analysis with specific antibodies. To verify equal loading, the blots were re-probed with an anti-β-actin antibody. The data are representative of 3 independent experiments.

To obtain further evidence for the induction of ER stress, we assessed the levels of 2 unfolded protein response (UPR) proteins GRP78 and XBP-1, after H_2O_2 treatment. Western blot analysis showed that treatment with the positive control thapsigargin considerably upregulated the expression of GRP78 for 24 h, while H_2O_2 treatment did not (Fig. 4D). On the other hand, H_2O_2 increased the expression of XBP-1, although the degree varied considerably in different experiments. Both the active spliced form (XBP-1s) and inactive unspliced form (XBP-1u) of XBP-1 were increased, and these effects were totally inhibited by MnTBaP treatment (Fig. 4E). Collectively, these data show that H_2O_2 induces ER stress responses and that scavenging of O_2^- inhibits them.

H_2O_2 induces minimal apoptosis and O_2^- generation in primary melanocytes. We examined the cytotoxic effect of H_2O_2 on

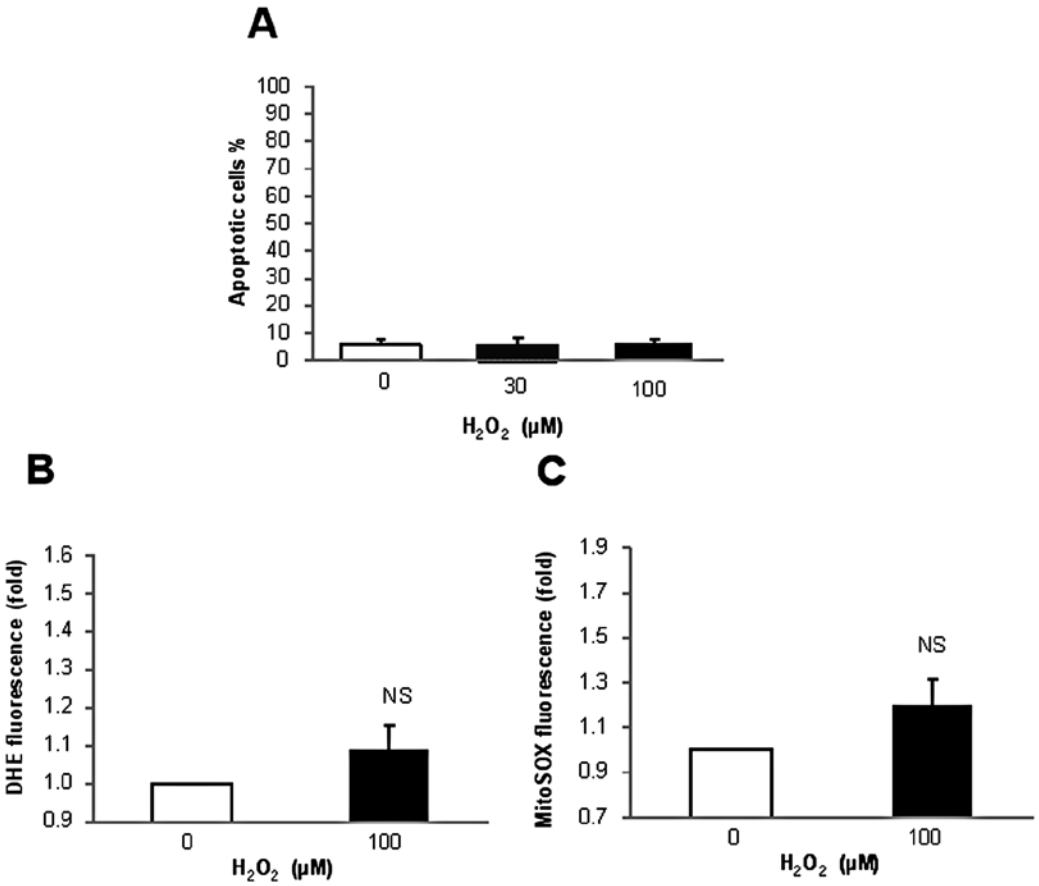

Figure 5. H_2O_2 induces minimal apoptosis and O_2^- generation in normal primary melanocytes. (A) Melanocytes were treated with 30 or 100 μM H_2O_2, stained with Annexin V/PI, and analyzed for apoptotic cell death by flow cytometry. The data are representative of 4 independent experiments. Melanocytes loaded with (B) DHE or (C) MitoSOX Red were treated with 100 μM H_2O_2 for 4 h and analyzed for fluorescence by flow cytometry. The data represent means ± SE from 4 independent experiments. NS, not significant.

primary normal melanocytes. Fluorescence microscopic analysis revealed that treatment with 100 ng/ml TRAIL and 100 μM H_2O_2 alone or in combination for 24 h resulted in minimal cell death (data not shown) and apoptosis (Fig. 5A) in normal melanocytes. In addition, only minimal intracellular and mitochondrial O_2^- generation was observed after 4-h H_2O_2 treatment (Fig. 5B and C). These data indicate that melanocytes are resistant to H_2O_2-induced cell death and O_2^- generation.

Discussion

In the present study, we investigated the possible role of H_2O_2 in TRAIL-induced apoptosis. TRAIL induced no or only a marginal increase in intracellular H_2O_2 levels in human TRAIL-resistant melanoma cells. On the other hand, exogenously applied H_2O_2 at relatively low concentrations (30-100 μM) substantially killed these cells. In addition, under certain circumstances, a synergistic induction of apoptosis was observed when H_2O_2 and TRAIL were applied in combination. Collectively, these data indicate that H_2O_2 is a modulator rather than primary mediator of the cytotoxic effect of TRAIL. Interestingly, the synergism was more clearly observed with low concentrations of H_2O_2 than with high concentrations of H_2O_2, suggesting that as the concentration increases, in addition to its intrinsic mechanism, H_2O_2 also stimulates apoptotic pathways that are at least partially shared with TRAIL. H_2O_2 induced apoptotic or necrotic cell death, depending on the concentration of the oxidant applied. The intrinsic mitochondrial pathway is considered to be the major mechanism of apoptosis. Consistent with this view, the cell death induced by low concentrations of H_2O_2 was caspase-dependent and was associated with increased $\Delta\Psi_m$ collapse and caspase-3/7 activation. However, inhibition of caspase-3/7 only partially blocked apoptosis. These data suggest that while the intrinsic mitochondrial pathway does play a role in inducing apoptosis, another caspase cascade may also be involved in this caspase-dependent apoptosis.

ER can initiate cell death through a pathway that is independent of intrinsic (mitochondria) and extrinsic (death receptor) pathways. ER-associated cell death is thought to be mediated by caspase-12 (22-26). A variety of cellular conditions such as glucose deprivation, hypoxia, disturbance of calcium homeostasis and excess ROS can cause ER stress, which is characterized by the accumulation of unfolded proteins. ER stress activates the adaptive UPR, which protects cells owing to protein synthesis inhibition, chaperone protein upregulation and an increase in protein degradation. If UPR activation is not able to relieve ER stress, the cells undergo ER-mediated apoptosis (22-26). Upon ER stress, the chaperone molecule GRP78 dissociates from the transmembrane proteins, such as inositol requiring enzyme 1α (IRE1α) and activating transcription

factor 6 (ATF6). The free ATF6 translocates to the Golgi apparatus where it is activated. The active ATF6 in turn enters the nucleus and initiates the expression of the transcription factor XBP-1. Activated IRE1α splices the transcribed XBP-1 mRNA to allow translation of the mature XBP-1 protein, which acts as a transcription factor and mediates the transcriptional upregulation of numerous genes involved in ER function (20,21,23). Our data showed that H_2O_2 induced ER stress, as shown by caspase-12 activation and upregulated the expression of the mature XBP-1 protein. Furthermore, inhibition of caspase-12 strongly blocked the H_2O_2-induced apoptosis. Collectively, our data suggest that the ER-mediated apoptotic pathway involving caspase-12 plays a key role in H_2O_2-induced apoptosis.

Interestingly, while activation of caspase-12 following the induction of ER stress during apoptosis has been reported in various mammalian cells including mouse, rat, rabbit and cow (26), the role of caspase-12 in ER-mediated apoptosis of human cells is a matter of debate. This might be because the human caspase-12 gene contains several mutations that block its expression (27). Nevertheless, an increasing body of evidence suggests that a caspase-12-like protein exists and is activated in human cells following the induction of ER stress by divergent causes, including H_2O_2, cisplatin, tetrocarcin A and hyperthermia (12,28-33).

Recently, adaptation to ER stress was suggested to be a key driver of malignancy and resistance to therapy in cancer cells, including malignant melanoma cells, with GRP78 playing a key role in this adaptation (34,35). GRP78 expression is associated with tumor development and growth and is correlated with resistance to chemotherapeutic drugs such as cisplatin and adriamycin (34-36). In this study, thapsigargin substantially increased GRP78 expression, while H_2O_2 decreased GRP78 expression in melanoma cells; these cells were killed by H_2O_2, but not thapsigargin. On the other hand, GRP78 expression was minimally increased in thapsigargin-sensitive Jurkat leukemia cells (Inoue and Suzuki, unpublished data). GRP78 has been shown to exert its anti-apoptotic function by inhibiting caspase-4 or caspase-7 activity (36). However, caspase-4 appears to regulate H_2O_2-induced apoptosis negatively rather than positively, as inhibition of the enzymatic activity significantly enhanced the apoptosis. Given the structural similarity between caspase-4 and caspase-12, it is possible that GRP78 may also target caspase-12 to counteract ER-mediated apoptosis.

ROS levels are controlled by the antioxidant defense system, including the antioxidant enzymes manganese- or copper-zinc-containing superoxide dismutase, which catalyze the dismutation of O_2^- into H_2O_2, and catalase and glutathione peroxidase, which degrade H_2O_2. Our data showed that these enzymes had no effects on H_2O_2-induced cell death. H_2O_2 is a diffusible molecule that is readily transported across the cell membrane to the extracellular space. Consequently, scavenging of extracellular H_2O_2 by catalase may eventually result in a decrease in the intracellular H_2O_2 level. Therefore, the ineffectiveness of catalase to suppress H_2O_2-induced cell death suggests that H_2O_2 *in situ* plays a minor role in the cell death. The ineffectiveness of MnTBaP to enhance H_2O_2-induced cell death supports this view, since MnTBaP increased intracellular H_2O_2 levels. Instead, our data showed that O_2^- is a key mediator in H_2O_2-induced cell death. H_2O_2 induced persistent intracellular O_2^- generation at concentrations that effectively induced cell death. Consistent with the role of mitochondria as the most common source of ROS during apoptosis, H_2O_2 induced substantial mitochondrial $O_2^{\bullet-}$ generation. Moreover, MnTBaP, a cell permeable SOD mimetic, reduced the H_2O_2-induced mitochondrial O_2^- generation and cell death, In addition, MnTBaP blocked H_2O_2-induced ER stress responses such as caspase-12 and XBP-1 activation. Collectively, these data suggest O_2^- most likely derived from the mitochondria mediates ER-mediated apoptosis, thereby promoting H_2O_2-induced cell death.

In conclusion, we have demonstrated for the first time that H_2O_2 induces cell death in TRAIL-resistant human melanoma cells via intracellular O_2^- generation. Further studies on the mechanisms by which H_2O_2 induces this O_2^- generation are under way. Since melanoma cells are much more susceptible to oxidative cell death than normal primary melanocytes, H_2O_2 has therapeutic potential in the treatment of malignant melanoma.

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