Ingenol 3-angelate induces dual modes of cell death and differentially regulates tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis in melanoma cells

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Abstract

Ingenol 3-angelate (PEP005), one of the active ingredients in an extract from *Euphorbia peplus*, was shown in preclinical studies to have activity against human melanoma xenografts in nude mice. In the present study, we have tested its ability to induce the apoptosis of melanoma cells *in vitro* in the absence or presence of tumor necrosis factor-related apoptosis inducing ligand (TRAIL). The results showed that at relatively high concentrations (100 μg/mL), PEP005 killed melanoma cells mainly by induction of necrosis. In 20% of cell lines, evidence of apoptosis was observed. Apoptosis was caspase-dependent and associated with changes in mitochondrial membrane potential that were not inhibitable by overexpression of Bcl-2 or inhibition of caspases but were blocked by inhibition of protein kinase C (PKC). Low concentrations (1 or 10 μg/mL) of PEP005 either increased or decreased TRAIL-induced apoptosis in a cell line–dependent manner. These changes in TRAIL-induced apoptosis seemed to be due to activation of PKC and varying levels of PKC isoenzymes in different melanoma cell lines. PEP005-mediated enhancement of apoptosis seemed to be associated with low expression of the PKCε isoform. These results indicate that PEP005 may enhance or inhibit sensitivity of melanoma to treatments associated with TRAIL-induced apoptosis depending on the PKC isoform content of melanoma cells. [Mol Cancer Ther 2004;3(12):1651–8]

Introduction

Extracts of *Euphorbia peplus* have been used to treat cancer and warts for many centuries (1), including rodent ulcers, and cancers of the stomach, liver, and uterus (2). More recently, it was shown to have activity against basal cell carcinoma and actinic lesions (3, 4). The mechanism of action of the extracts is largely unknown. An active fraction, Ingenol 3-angelate (PEP005), was shown to induce differentiation of certain cultured melanoma cells to bipolar or dendritic cell morphology and at higher doses, death of the melanoma cell lines (5). In mice studies involving topical application of PEP005, it was shown to induce regression in numerous subcutaneous tumors. Disruption of the plasma membrane and loss of mitochondrial membrane potential (MMP) followed by rapid necrotic death of cells was observed in these studies (5).

Over the past decade, it has become known that many therapeutic agents kill cancer cells by inducing apoptosis (6–8). Two main pathways are involved. One involves interaction of ligands belonging to the tumor necrosis factor family with corresponding receptors on the cell surface, which initiates activation of caspases leading to apoptosis. The most important of these ligands is tumor necrosis factor-related apoptosis inducing ligand (TRAIL), which interacts with two death-inducing receptors on the cell surface, referred to as TRAIL-R1 (DR4) and TRAIL-R2 (DR5; refs. 6, 9).

The second pathway to apoptosis is the intrinsic pathway, which depends on damage to mitochondria and release of apoptosis-inducing proteins from mitochondria. Agents that damage DNA, or the cytoskeleton of the cell, mediate this form of apoptosis by up-regulation and activation of proapoptotic ''BH3-only'' members of the Bcl-2 family of proteins such as PUMA, Noxa, Bim, and Bmf (10). Clear distinction between these two pathways is not always possible as receptor ligand interactions can also cause activation of a BH3-only protein called Bid, which activates the mitochondrial pathway to cell death (9, 11). In the case of TRAIL-mediated killing of melanoma, this was shown to be the principal pathway to cell death (11, 12).

The purpose of the present studies was to examine whether PEP005 induced apoptosis of melanoma cells, and in particular, whether PEP005 sensitized melanoma cells to apoptosis induced by TRAIL. The results show that PEP005 can induce apoptosis in some melanoma cell lines but the predominant form of cell death is nonapoptotic. Further, PEP005 has complex effects when tested in combination with T+RAIL, which may be associated with expression of different protein kinase C (PKC) isoforms in the melanoma cell lines.

Materials and Methods

Cell Lines

Human melanoma cell lines, Me4405, Me1007, IgR3, Mel-FH, Mel-RMu, Mel-RM, Mel-CV, Mel-AT, MM200, and

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Sk-Mel-28 have been described previously (13). The cell lines were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Australia). Melanocytes were kindly provided by Dr. P. Parson (Queensland Institute of Medical Research, Brisbane, Australia) and cultured in medium supplied by Clonetics (Edward Kellar, Victoria, Australia).

PEP005, Antibodies, Recombinant Proteins, and Other Reagents

PEP005 was supplied by Peplin Biotech (Brisbane, Queensland, Australia). It was prepared from *Euphoria peplus* and the purity of this fraction was greater then 95% by high-performance liquid chromatography (5). Recombinant human TRAIL was supplied by Immunex (Seattle, WA). The preparation was supplied as a leucine zipper fusion protein, which required no further cross-linking for maximal activity. The mouse monoclonal antibody (MAb) against TRAIL-R1 and TRAIL-R2 were supplied by Immunex and have been described previously. The rabbit antiserum against the inhibitor of caspase-activated deoxyribonuclease (ICAD) and the control preimmune serum were a kind gift from Dr. S.L. Sabol (National Cancer Institute, Bethesda, MD) and were described elsewhere (14). The rabbit polyclonal antibody (Ab) against caspase-3, caspase-8, Bid, the mouse MAbs against cytochrome *c*, and PARP were from Pharmingon (San Diego, CA). Rabbit polyclonal Abs against c-IAP1, c-IAP2, and A1, n-PKCδ, n-PKCε, phosphorylated n-PKCδ, phosphorylated n-PKCε and mouse MAbs against Bcl-2, Bcl-X_L and Mcl-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse MAb against phosphorylated Erk1/2 was also purchased from Santa Cruz Biotechnology. The mouse polyclonal Ab against Bax was supplied by Oncogene Research Products (Cambridge, MA). The mouse MAb against X-linked inhibitor of apoptosis protein was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-Bax against amino acids 1 through 20 were purchased from Upstate Biotechnology (Lake Placid, NY). The MAb against cytochrome *c* oxidase subunit 4 (COX IV) was purchased from Molecular Probes (Eugene, OR, USA). Isotype control Abs used were the ID4.5 (mouse IgG2a) MAb against *Salmonella typhi* supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, Australia), the 107.3 mouse IgG1 MAb purchased from PharMingen (San Diego, CA), and rabbit IgG from Sigma Chemical Co. (Castle Hill, Australia). The general caspase inhibitor, Z-Val-Ala-Asp(Ome)-CH_2F (z-VAD-fmk), the caspase-8 inhibitor, Z-Iie-Glu(Ome)-Thr-Asp(Ome)-CH_2F (z-IETD-fmk), and the caspase-9 inhibitor, Z-Leu-Glu(Ome)-His-Asp(Ome)-CH2F (z-LEHD-fmk) were purchased from Calbiochem (La Jolla, CA).

Plasmid Vectors

The expression construct of pEF Bcl-2 was a kind gift from Dr. David Vaux (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), which was transfected into melanoma cells by electroporation and the resulting transfectants were maintained as described previously (11, 15).

Flow Cytometry

Immunostaining on intact and permeabilized cells was carried out as described previously (13). Analysis was carried out using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer. The percentage of antigen-positive cells was calculated as the difference in positive area between the positive and negative control histograms. The positive area was that to the right of the intersection of the two curves (11).

Apoptosis

Apoptotic cells were determined by staining using the propidium iodide (PI) method described elsewhere (13). In brief, melanoma cells were adhered overnight in a 24-well plate (Falcon 3047; Becton Dickinson, Lane Cove, Australia) and treated with TRAIL or PEP005. Floating and adherent cells were then harvested and incubated overnight at 4°C in the dark with 750 μl of a hypotonic buffer (50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma) before flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson).

Cell Viability

Cell viability was measured by examination of flow cytometric staining patterns obtained with PI and staining with FITC-conjugated Annexin V according to the manufacturer's instructions and as described elsewhere (16). In brief, cells with or without pretreatment with PEP005 or TRAIL were washed twice with cold PBS, resuspended in binding buffer and stained with Annexin V-FITC. After incubation at room temperature for 15 minutes in the dark, an additional 400 μl of binding buffer was added to each tube, and cells were analyzed by flow cytometry within 1 hour. Cells positive for PI only are necrotic, and cells positive for Annexin V only are in the early stages of apoptosis. Cells that are positive for both PI and Annexin V may be necrotic or in the late stages of apoptosis.

Mitochondrial Membrane Potential

Methods used were similar to those described previously (17). Tumor cells were cultured in 24-well plates and allowed to reach exponential growth for 24 hours before treatment. MitoTracker Red CMXRos (Molecular Probes) was added at 100 nmol/L during the last 30 minutes of treatment. The medium was removed into a 75-mm Falcon polystyrene tube and the adherent cells were trypsinized and collected into the same tube. After washing with PBS, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) for mitotracker uptake. Untreated cells were used as controls.

Western Blot Analysis

Methods used were as described previously (11), with minor modification. Cellular protein (20 μg) was electrophoresed on 10% to 15% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked, incubated with primary Abs at the appropriate concentration, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution; Bio-Rad, Hercules, CA). Labeled bands were detected by

Renaissance Western blot chemiluminescence reagent (New England Nuclear Life Science Products, Boston, MA) and exposed on Hyper MP autoradiography film (Amersham, Piscataway, NJ).

Results

PEP005 Induces Both Nonapoptotic and Apoptotic Cell Death

Figure 1A illustrates morphologic changes induced by PEP005 in Mel-RM and IgR3. As early as 1 hour after the addition of PEP005 at 100 μg/mL, the cytosol became granular in appearance and the volume was gradually reduced over 6 hours until the nucleus constituted the majority of the cellular volume. The cells also lost the ability to adhere to the plates. The impact of PEP005 on cell viability was quantitated by staining with Annexin V and PI. The dose and time dependence of reduced viability is shown in Fig. 1B. Flow cytometry detection of DNA fragmentation induced by PEP005 in the cell line Me1007 but not in Mel-RM is shown in Fig. 1C. Only two of the cell lines tested had significant levels of apoptosis (Me1007 and Me4405), and the relative contributions of apoptosis and nonapoptotic cell death in the melanoma cell lines is shown in Fig. 1D.

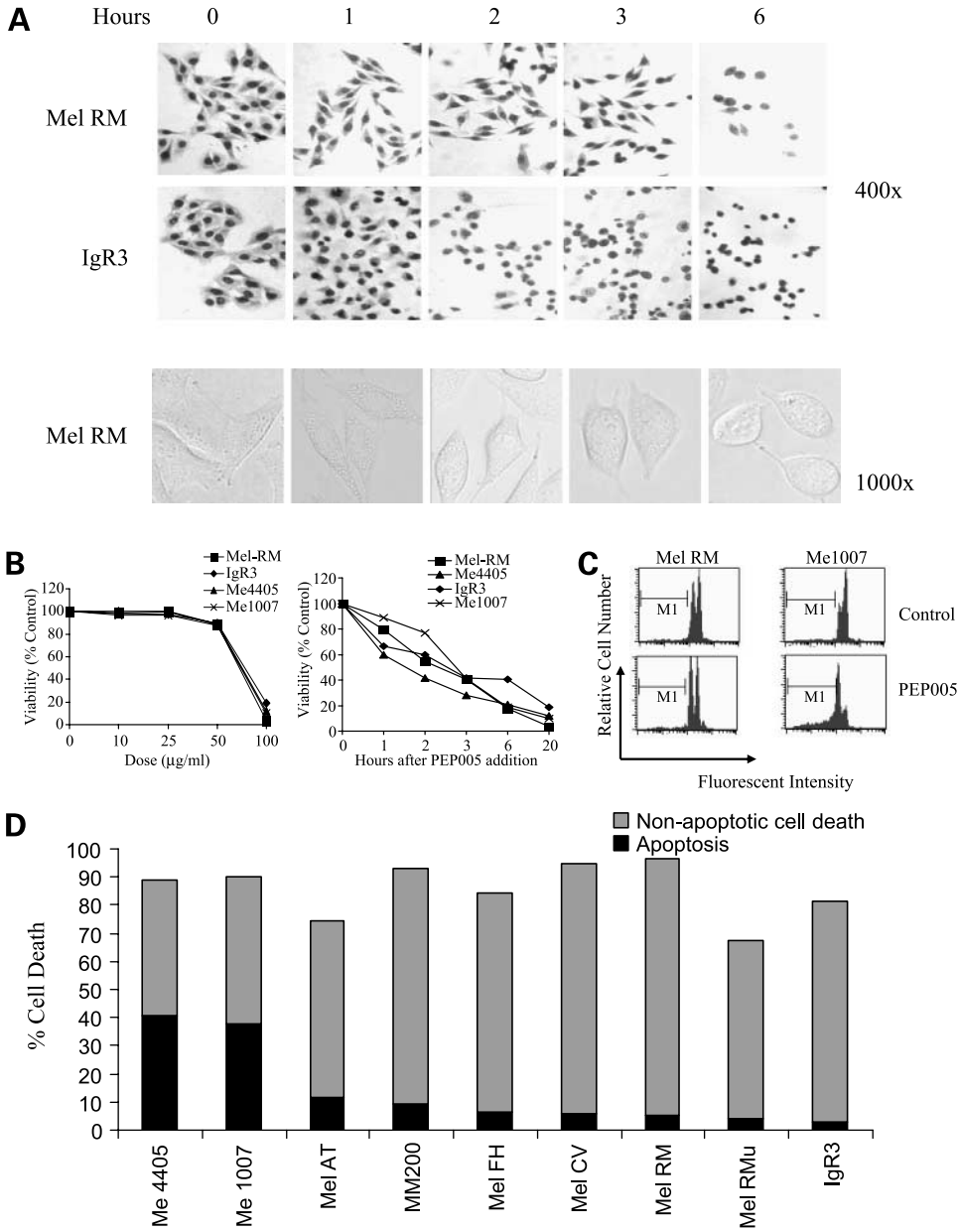

Figure 1. PEP005 induces morphologic changes and affects the viability of cultured melanoma cells. **A,** cell lines Mel-RM and IgR3 under 400× magnification treated with PEP005 at 100 μg/mL and stained to show nuclear and cytoplasmic volume; microphotographs of PEP005-treated (100 μg/mL) Mel-RM under 1,000× magnification. **B,** cells were treated with different concentrations of PEP005 and stained with Annexin V and PI to determine cell viability. The maximum amount of cell death was induced at 100 μg/mL and peaked at 20 hours. **C,** flow cytometry histograms of apoptosis assays by the PI method. Mel-RM and Me1007 were treated with 100 μg/mL of PEP005 overnight. **D,** treatment with PEP005 (100 μg/mL) affected the viability of all melanoma cell lines but apoptosis only contributed significantly in two of the cell lines tested. The percentage of nonviable cells was measured with Annexin V-FITC and PI dual staining by flow cytometry. The proportion of apoptotic cells was measured with PI staining of DNA fragmentation by flow cytometry.

PEP005-Induced Apoptosis Is Caspase-Dependent and Associated with Changes in Mitochondria That Are Blocked by Inhibition of PKC

Two apoptosis-sensitive and two resistant melanoma cell lines were pretreated with a pan-caspase inhibitor, z-VAD-fmk, and specific inhibitors of caspase 9 (z-LEHD-fmk) and caspase 3 (z-DEVD-fmk). As shown in Fig. 2A and B, the pan-caspase inhibitor and inhibitor of caspase 9 almost completely inhibited apoptosis in the Me4405 and Me1007 cell lines, whereas there was only partial inhibition with the inhibitor of caspase 3 (Fig. 2C). Caspase 3 was however activated (Fig. 2D), as shown by flow cytometric detection with a MAb specific for the activated form of caspase 3 (11). Even though the caspase inhibitors inhibited apoptosis, the same morphologic changes associated with PEP005 treatment noted in Fig. 1 were still observed in all cell lines tested.

Possible involvement of mitochondria in PEP005-induced cell death was examined by studying changes in MMP. Exposure of both the PEP005 apoptosis-sensitive Me4405 cells and the PEP005-resistant Mel-RM cells resulted in a decrease in MMP (Fig. 3A). Figure 3B shows that overexpression of Bcl-2 inhibited TRAIL-induced changes in MMP but had no effect on the changes in MMP resulting from treatment with PEP005. We further examined whether caspase-dependent or PKC-dependent pathways may be involved in PEP005 induced changes in MMP. As shown in Fig. 3C, the changes in MMP induced by PEP005 were completely inhibited by pretreatment of the Mel-RM cell line with the PKC inhibitor bisindolylmaliamide, however, no inhibition was seen with the pan-caspase inhibitor z-VAD-fmk. Similar results were seen in Me4405 (data not shown).

PEP005 Differentially Regulates Sensitivity of Melanoma Cells to TRAIL-Induced Apoptosis

To test the effect of PEP005 on TRAIL-induced apoptosis, we added PEP005 at 1 and 10 μg/mL to assays with TRAIL at 200 ng/mL. At 10 μg/mL, apoptosis was increased in Me4405, Mel-AT, and IgR3, but decreased in Mel-CV, Mel-RM, MM200, and Mel-FH. However, at 1 μg/mL, Me4405 was the only cell line observed to have an increase in TRAIL-induced apoptosis (Fig. 4A and B).

To determine the mechanism by which PEP005 was affecting the level of TRAIL-induced apoptosis, we tested its effect on the level of expression of TRAIL death receptors in the melanoma cell lines. As seen in Fig. 5A, there were no change in the level of the two death receptors, TRAIL-R1 or TRAIL-R2, with PEP005 treatment. Cleavage of caspase 8 was determined by Western blot with an Ab specific for procaspase 8. As seen in Fig. 5B, PEP005 alone had no effect on the level of procaspase 8, and also had no effect on the amount of cleavage induced by TRAIL.

PEP005 at Low Concentrations Reduces or Increases TRAIL-Mediated Changes in Mitochondria and Downstream Events Depending on the Cell Line

To better understand the effects of PEP005 on TRAIL-mediated apoptosis, we examined changes in MMP after exposure to TRAIL for 3 hours preceded by overnight treatment with PEP005 at 1 μg/mL. TRAIL-induced changes in MMP were increased in the Me4405 cells by pretreatment with PEP005 but decreased in the Mel-RM cell line which was inhibited by PEP005 (Fig. 5C). Similar results were obtained in a repeat of the experiment. Changes in TRAIL-mediated caspase 3 activation were consistent with the observed changes in MMP in that activation of caspase 3 was enhanced by PEP005 at 1 μg/mL in Me4405 cells and decreased in Mel-RM cells (Fig. 5D and E). There was a corresponding decrease in degradation of ICAD, a substrate of caspase 3, in the Mel-RM cells when PEP005 and TRAIL were used together, compared with TRAIL alone.

The Effect of PEP005 on TRAIL-Induced Apoptosis Is Blocked by Inhibition of PKC

The PKC inhibitor, bisindolylmaleimide, was added to melanoma cell lines for 1 hour prior to treatment with TRAIL, with or without PEP005 at 10 μg/mL, to determine if PKC activation by PEP005 played a role in the effect of PEP005 on TRAIL-induced apoptosis. As shown in Fig. 6A,

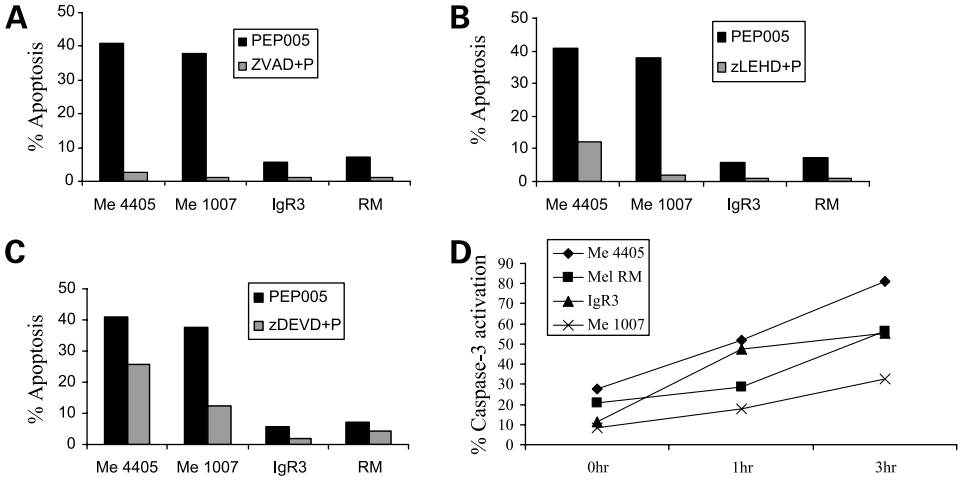

Figure 2. Apoptosis induced by PEP005 is caspase-dependent. Four cell lines were treated with (**A**) a pan-caspase inhibitor, z-VAD-fmk, (**B**) a caspase-9 inhibitor, z-LEHD-fmk, or (**C**) a caspase-3 inhibitor, z-DEVD-fmk for 1 hour prior to overnight treatment with PEP005 at 100 μg/mL. Apoptosis was measured using the PI method using flow cytometry. **D,** the activation of caspase-3 was also measured after PEP005 treatment via flow cytometry with an antibody specific for the activated form of caspase-3.

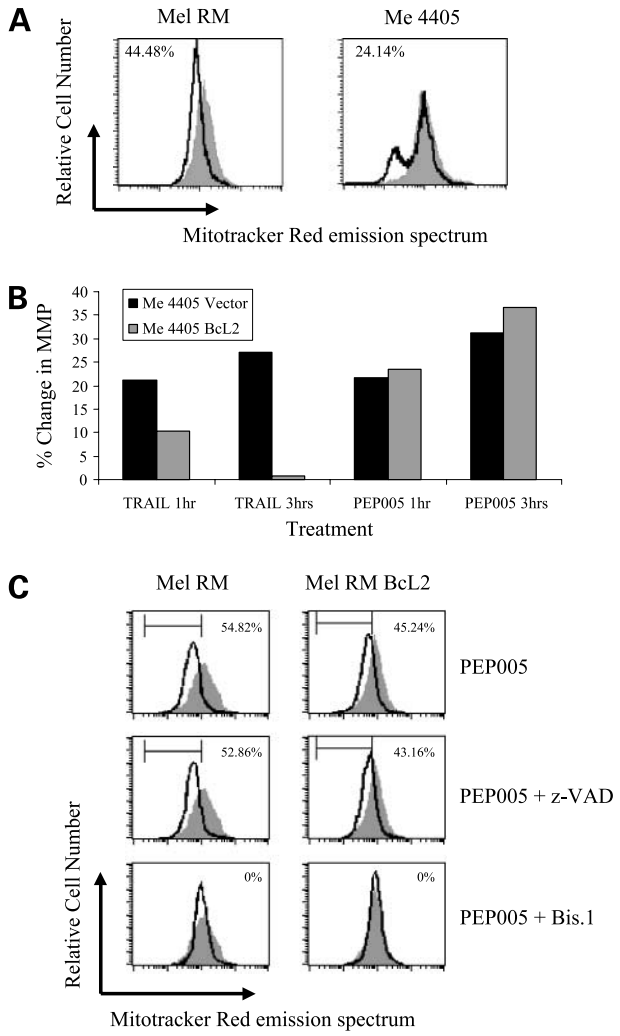

Figure 3. PEP005 induces changes in mitochondrial membrane permeability. **A,** representative flow cytometry histograms of the change in MMP induced by PEP005. Mel-RM and Me4405 were treated for 3 hours with 100 μg/mL of PEP005 and the change in MMP measured by the uptake of MitoTracker Red CMXRos. *Closed histograms,* untreated control cells; *open histograms,* cells treated with PEP005. The percentage shown is the reduction in the treated cells compared with the control. **B,** change in MMP was measured after treatment with PEP005 at 100 μg/mL in Me4405 cells overexpressing BcL-2. TRAIL was used as a positive control for inhibition. **C,** cells were treated with the pan-caspase inhibitor, z-VAD-fmk or the PKC inhibitor, bisindolylmaleimide 1 for 1 hour prior to treatment with PEP005 at 100 μg/mL for 3 hours. *Filled histogram,* untreated cells; *open histogram*, treated cells. The percentages indicate the reduction in MMP in the treated cells compared with the control.

the inhibition of TRAIL-induced apoptosis by PEP005 in Mel-RM is reversed in cells treated with the inhibitor of PKC. The PKC inhibitor also reversed the PEP005 mediated increase in TRAIL-induced killing of Me4405.

PEP005-Mediated Regulation of TRAIL-Induced Apoptosis Is Affected by Different Expression Patterns of PKC Isoforms within Cells

To further examine the role of PKC in PEP005 and TRAIL-induced apoptosis, the level of expression and phosphorylation of two different isoforms, PKCδ and PKCε, was tested before and after treatment with PEP005 and TRAIL both individually and together. The most striking trend seen in the cell lines is the comparatively lower constitutive levels of the antiapoptotic PKCε (Fig. 6C), compared with PKCδ (Fig. 6B), in Me4405 and Mel-AT, two cell lines that were sensitized to TRAIL by PEP005 (10 μg/mL). Furthermore, no phosphorylation of PKCε was seen in these cell lines prior to or following treatment. Note also that PKCε and phosphorylated PKCε were at low levels before and after treatment in IgR3 cells and that TRAIL-induced apoptosis was also increased in the presence of PEP005 in this cell line (Fig. 4A). However, there was clearly an up-regulation of phosphorylated PKCδ in Me4405 and Mel-AT following treatment with TRAIL and PEP005. Similar up-regulation of phosphorylated PKCδ was seen in Mel-RM but this was coupled with much higher levels of PKCε and phosphorylated PKCε in this cell line, both before and after treatment, and is consistent with the observed suppression of TRAIL-induced apoptosis by PEP005 in this line.

Discussion

The above results indicate that at high concentrations, PEP005 induced cell death in all of the cultured melanoma cell lines tested. Apoptosis was observed in approximately 20% of the lines, the main form of cell death, however, was necrosis. The apoptotic portion of cell death was caspase-dependent and associated with changes in MMP. In contrast to changes in ΔΨm induced by TRAIL, PEP005-induced changes were not inhibitable by overexpression of Bcl-2. Bcl-2 is believed to protect mitochondria by binding to multidomain proteins Bax and Bak, which in turn, inhibit the action of BH3-only proapoptotic proteins, such as tBid (activated by TRAIL), Bim or Bmf, released from the cytoskeleton or Noxa and Puma due to up-regulation of p53 (10, 18). Failure of Bcl-2 overexpression to inhibit apoptosis therefore suggests that the activation of the proapoptotic BH3-only proteins was not involved in the induction of apoptosis, and is consistent with either a direct effect of PEP005 on mitochondria as suggested by others (5), or an effect mediated by activation of PKC. The latter was suggested by inhibition of changes in ΔΨm induced by PEP005 by inhibitors of PKC. This may indicate direct interaction of PKC isoenzymes with proapoptotic proteins such as Bax as reported for PKCε in prostate carcinoma cells (19).

Interactions of PEP005 and TRAIL were of particular interest in that TRAIL-mediated apoptosis was regulated differentially in different cell lines. Three cell lines showed increased levels of apoptosis when TRAIL was used in conjunction with PEP005 at 10 μg/mL, whereas the other cell lines were protected from TRAIL-induced apoptosis by cotreatment with PEP005. This effect was dose-dependent and only one cell line still showed increased levels of apoptosis with TRAIL and PEP005 at 1 μg/mL. The combination of both agents on apoptosis was associated with similar changes in MMP, i.e. cells in which TRAIL-induced

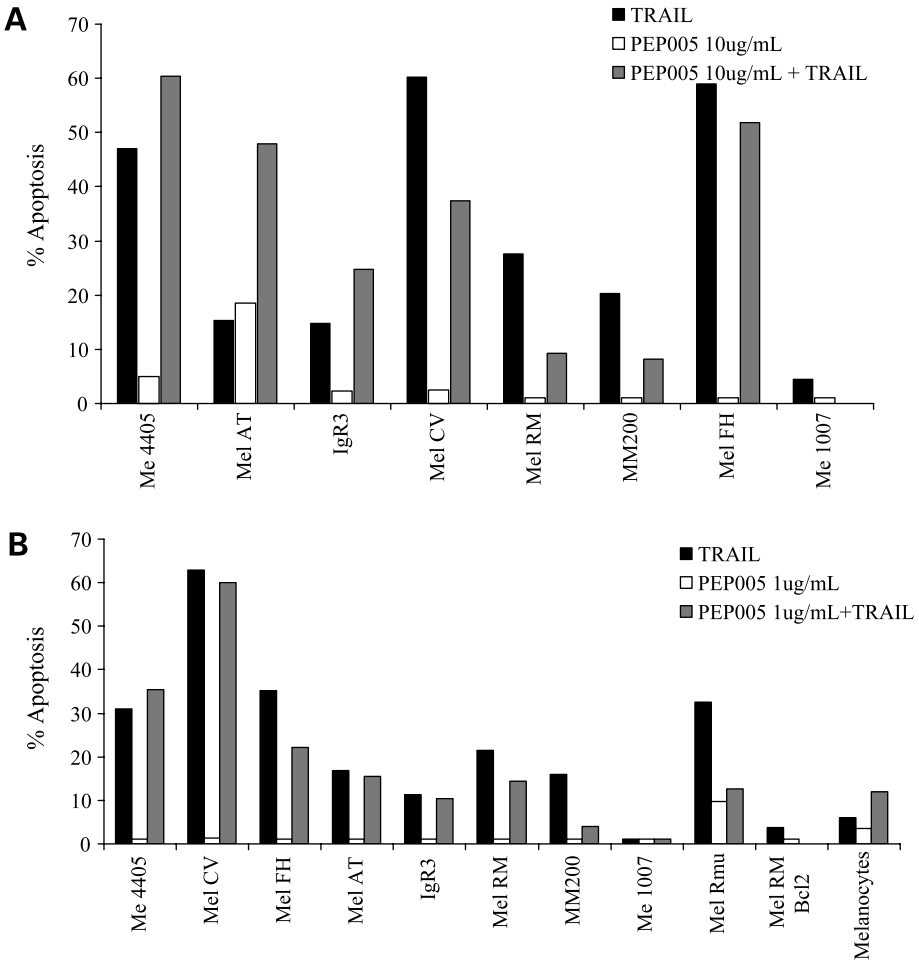

Figure 4. PEP005 sensitizes or protects melanoma cells from TRAIL-induced apoptosis in a cell line–dependent and dose-dependent manner. **A,** apoptosis induced by cotreatment with TRAIL at 200 ng/mL and PEP005 at 10 μg/mL; and **B,** 1 μg/mL in a panel of melanoma cell lines and melanocytes. Cells were treated overnight (16 hours).

apoptosis was increased showed increased changes in MMP and caspase 3 activation and the corresponding cleavage of ICAD compared with TRAIL treatment alone. The opposite was found in cell lines where PEP005 suppressed TRAIL-induced apoptosis.

Ogborne et al. (5) identified PKC as an important target for PEP005 and showed that inhibition of PKC by bisindolylmaleimide inhibited growth arrest and bipolar changes induced by PEP005. More recently, its association with PKC has been better defined (20). In view of this, we investigated the effect of this PKC inhibitor on PEP005-induced changes in TRAIL-induced apoptosis. Inhibition of PKC in Mel-RM, a cell line in which PEP005 inhibited TRAIL-induced apoptosis, reversed the inhibitory effect of PEP005. Inhibition of PKC in Me4405 on the other hand reversed the increase of TRAIL-induced apoptosis mediated by PEP005 treatment. Further investigation showed that expression and phosphorylation patterns of proapoptotic (PKCδ) and antiapoptotic (PKCε) isoforms of PKC varied widely between cell lines. Most significantly, the expression of PKCδ, an antiapoptotic member of the PKC family (21) was shown to have low expression in the cell lines Me4405 and Mel-AT, in which PEP005 increased TRAIL-induced apoptosis. There was also low or no detectable phosphorylation of PKCε after treatment with TRAIL, PEP005, or the combination of both in Me4405 cells.

To our knowledge, the present study is the first to draw attention to the variability in expression of different PKC isoforms between different melanoma lines and the importance of PKC isoenzyme content on susceptibility to TRAIL-induced apoptosis of melanoma cells. Studies by others on pancreatic cell lines have shown that TRAIL may activate PKC (22) and that activation of PKC may inhibit TRAIL-induced killing of Jurkat T cells (23). Activation of PKCε was reported to be responsible for resistance to TRAIL-induced apoptosis of glioma cells (24).

Just how PKCε may inhibit apoptosis is not clear. It was reported to interact directly with the permeability transition pore in mitochondria of cardiac muscle (25) and to promote survival of lung carcinoma cells by inhibiting release of proapoptotic proteins from the mitochondria (23). It was also reported to directly associate with ERK1/2 kinases (26) or with Bax (19). PKCδ on the other hand was associated with the induction of apoptosis (27). The present

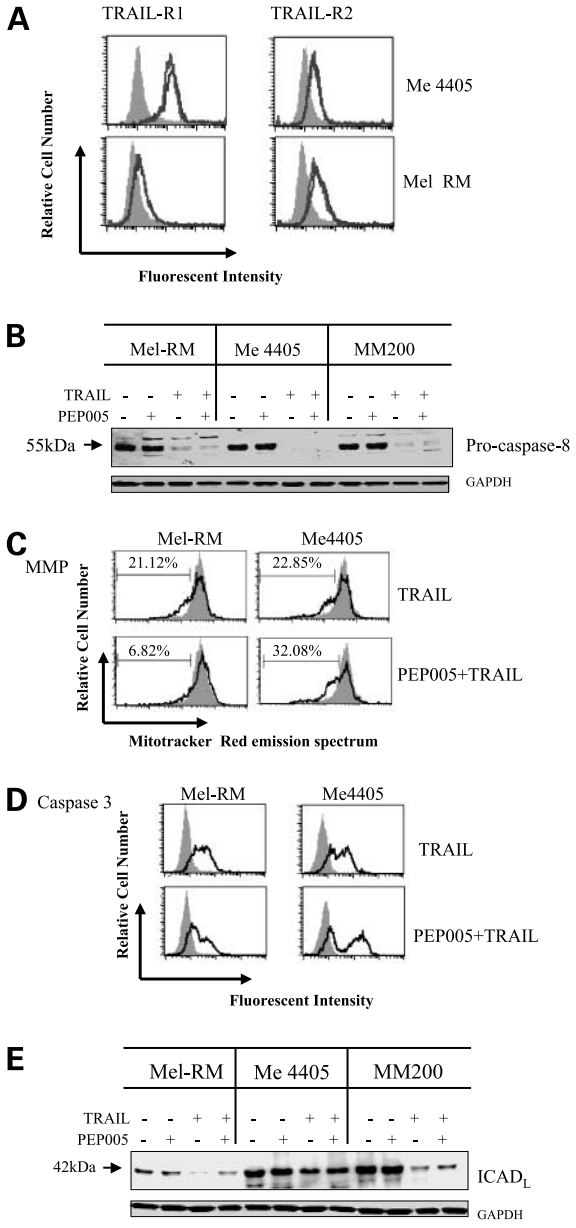

Figure 5. PEP005 does not affect the level of TRAIL-R1 or TRAIL-R2 expression but differentially regulates TRAIL-induced changes in MMP and caspase-3 activation. **A,** histograms of flow cytometric analysis of TRAIL-R1 and TRAIL-R2 expression. Cells were treated with PEP005 at 10 μg/mL and harvested for staining. *Closed histogram,* negative control; *open histograms,* treated and untreated cells. **B,** Western blot analysis of procaspase-8 cleavage. Cells were treated with PEP005 at 10 μg/mL overnight and then for a further 3 hours with TRAIL at 200 ng/mL and harvested for whole-cell lysate. **C,** histograms of flow cytometric analysis of changes in MMP measured by the uptake of MitoTracker Red CMXRos. Cells were treated with PEP005 at 10 μg/mL and TRAIL at 200 ng/mL. *Closed histograms,* untreated cells; *open histograms,* treated cells. The percentage shown in the difference between the treated and control cells. **D,** cells were treated with TRAIL at 200 ng/mL and PEP005 at 10 μg/mL for 3 hours, cells stained with an antibody specific for activated caspase-3 and analyzed by flow cytometry. **E,** Western blot analysis of cleavage of ICAD. Mel-RM, MM200, and Me4405 were treated with TRAIL at 200 ng/mL and PEP005 at 10 μg/mL and harvested for whole-cell lysate.

study shows that both PEP005 and TRAIL activate PKC isoforms that act to reduce or enhance changes in MMP induced by TRAIL depending on the relative content of these PKC isoforms in the cells.

In summary, PEP005 in high doses may initiate necrosis and apoptosis, or necrosis alone, in a cell line–dependent manner. At low doses, it may increase or decrease apoptosis induced by TRAIL depending on the cell line under study. The outcome of these interactions seems to depend on the PKC isoform content of the melanoma cells. In cells with

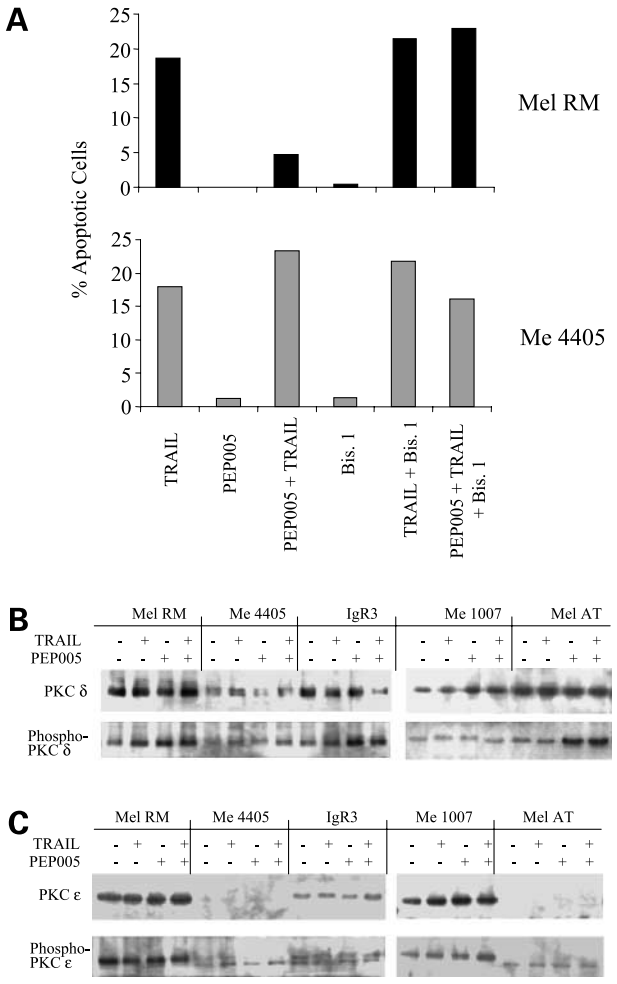

Figure 6. The effect of PEP005 on TRAIL-induced apoptosis is changed by inhibition of PKC. **A,** Mel-RM and Me4405 were treated with TRAIL at 200 ng/mL and PEP005 at 10 μg/mL overnight preceded by 1 hour of treatment with the protein kinase inhibitor, bisindolylmaleimide 1 (20 μM), and apoptosis was analyzed using the PI method and flow cytometry. The results shown are representative of two independent experiments. Variations in PKCδ and PKCε expression between melanoma cell lines. **B,** Western blot analysis of the expression of phosphorylated and non-phosphorylated PKCδ. Cells were treated with TRAIL at 200 ng/mL or PEP005 at 10 μg/mL or both compounds together and whole-cell lysate harvested. (*Bottom band*) the specific band for PKCδ. **C,** Western blot analysis of the expression of phosphorylated and nonphosphorylated PKCε. Cells were treated with TRAIL at 200 ng/mL or PEP005 at 10 μg/mL or both compounds together and whole-cell lysate harvested. *Top band*, the specific band for PKCε.

high levels of antiapoptotic isoforms, PEP005 may induce necrosis by direct effects on mitochondria. In those with low antiapoptotic isoforms, death is due to both apoptosis and necrosis. When apoptosis is induced by TRAIL, PEP005 at low doses, increases or decreases apoptosis depending on the levels of antiapoptotic PKC isoforms in the cells. The present results seem to have implications for treatment of melanoma with PEP005 alone or in combination with treatments that depend on TRAIL-induced apoptosis. Depending on the relative content of PKC isoforms in melanoma of individual patients, PEP005 could enhance or inhibit sensitivity of melanoma to treatment with TRAIL. Combination with TRAIL may also increase toxicity against normal cells, such as melanocytes, as shown in the present study.

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