Crosstalk between Extrinsic and Intrinsic Cell Death Pathways in Pancreatic Cancer: Synergistic Action of Estrogen Metabolite and Ligands of Death Receptor Family

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Abstract

2-Methoxyestradiol is a physiologic metabolite of 17β-estradiol. This orally active compound can inhibit tumor growth or metastasis in tumor models without inducing any clinical sign of toxicity. Our previous studies indicated that 2-methoxyestradiol-mediated apoptosis involves the disappearance of intact 21-kDa Bid protein, cytochrome c release, and predominant procaspase-3 cleavage. Here, using MIA PaCa-2 cells as a model, we investigated whether this estrogen metabolite induces apoptosis by converging two major pathways: the death receptor–mediated extrinsic and the mitochondrial intrinsic pathway. Exogenous expression of dominant-negative caspase-8 or dominant-negative FADD reverts the effect of 2-methoxyestradiol-mediated cell death. In parallel with this observation, Z-IETD-FMK, a cell permeable irreversible inhibitor of caspase-8, can render significant protection against 2-methoxyestradiol-induced apoptosis. RNase protection assay and cell surface receptor analysis by flow cytometry show the up-regulation of members of death receptor family in 2-methoxyestradiol-exposed pancreatic cancer cells. Our mechanistic studies also implicate that oxidative stress precedes 2-methoxyestradiol-mediated c-Jun NH2-terminal kinase activation, leading to elevated Fas level. Because 2-methoxyestradiol is able to trigger death receptor signaling, we were interested in examining the effects of 2-methoxyestradiol and Fas ligand (Fasl)/tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) together on pancreatic cancer cell death. Interestingly, the endogenous angiogenesis inhibitor 2-methoxyestradiol augments Fasl/TRAIL–induced apoptosis in these cells. Moreover, the combination of 2-methoxyestradiol and TRAIL reduces the tumor burden in vivo in MIA PaCa-2 tumor xenograft model by caspase-3 activation. (Cancer Res 2006; 66(8): 4309-18)

Introduction

Pancreatic carcinoma stands out as a highly lethal disease and is one of the leading causes of cancer-related death. Due to its metastasis-prone and locally resistant nature, the prognosis is very poor. To improve therapeutic outcome, it is necessary to identify novel agents or combination therapy that might be helpful in treating pancreatic cancer patients with fewer side effects.

Cancer cells acquire the ability to penetrate into lymphatic and blood vessels, circulate through the bloodstream, and then invade to grow in normal tissues elsewhere. In large measure, its invasive nature to other tissues and organs makes cancer a potentially life-threatening disease. The metastasis of cancer requires angiogenesis (1, 2). The angiogenic agents disrupt this process, thus cutting off the tumor’s supply of oxygen and nutrients (3). Such an angiogenesis inhibitor, 2-methoxyestradiol is an endogenous metabolite of 17β-estradiol formed by the sequential hydroxylation and methylation at the 2-position. A number of studies have shown that 2-methoxyestradiol can suppress growth of tumor cells in vitro and in vivo (4–18) by the induction of a cellular program called apoptosis or programmed cell death.

Programmed cell death or apoptosis is a highly regulated process that is essential for the development and tissue homeostasis within all multicellular organisms (19, 20). Diverse stimuli, such as growth factor withdrawal, deregulation of the cell cycle, DNA damage (19, 20), or death receptor-ligand interaction (21), might be responsible for initiating the apoptotic cascade. These proapoptotic signals induce several early events that converge by activating a common biochemical pathway, which then leads to the execution of apoptosis. Mitochondrion appears to be a core component of the cell death machinery (20, 22). Several cell death signals have been reported to release cytochrome c from mitochondria into the cytosol (20, 22). Released cytochrome c then binds to apoptosis protease-activating factor-1 to form an apoptosome complex with procaspase-9 in an energy-dependent manner (20, 22). Subsequently, activated caspase-9 triggers downstream caspases, such as caspase-3 and caspase-7 (20, 22). These downstream caspases, through the cleavage of several death substrates, such as poly(ADP-ribose) polymerase (PARP), are believed to cause execution of cell death (4, 19, 20).

Among the two major apoptotic pathways, such as mitochondrial “intrinsic” and transmembrane “extrinsic,” the latter one comprises of activation of death receptors, such as Fas, tumor necrosis factor (TNF) receptor 1, DR4, or DR5 (23–25). Upon activation by interacting with their respective ligands, such as Fas ligand (Fasl)/TNF-related apoptosis-inducing ligand (TRAIL; ref. 26), a signal transduction cascade ensues by the recruitment of death receptor–associated molecules, such as Fas-associated death domain containing protein (FADD). Subsequent activation of initiator caspsases, such as caspase-8, can lead to cleavage of a proapoptotic protein Bid. Truncated Bid can translocate to mitochondria and can orchestrate mitochondrial events that can result in biochemical as well as morphologic alterations implicated with programmed cell death (20).

Previously, we observed the disappearance of an intact 21-kDa Bid protein, cytochrome c release, and predominant procaspase-3
cleavage in 2-methoxyestradiol-challenged pancreatic cancer cells (4). In the current report, we investigated whether activation of caspase-8 plays any role in the case of 2-methoxyestradiol-triggered apoptotic cell death in pancreatic carcinoma cells. Transient expression of dominant-negative caspase-8 (27) or dominant-negative FADD (28) can attenuate the effect of 2-methoxyestradiol-mediated cell death. In parallel with this observation, Z-IETD-FMK, a cell permeable irreversible inhibitor of caspase-8, can render significant protection against 2-methoxyestradiol-induced apoptosis. To understand the mechanism of caspase-8 activation, RNase protection assay (RPA) using multiprobe template (29) set indicates the up-regulation of Fas, a member of the death receptor family.

TRAIL is a type II transmembrane cytokine and a potent inducer of apoptosis in cancer cells (26, 28–31). Because 2-methoxyestradiol is able to trigger death receptor signaling, we were interested to examine the effects of 2-methoxyestradiol and TRAIL together in cell culture and in animal model. Interestingly, the estrogen metabolite 2-methoxyestradiol cooperates with Fasl and TRAIL, to sensitize pancreatic cancer cells to apoptosis in vitro. To determine the synergistic interactions of 2-methoxyestradiol and TRAIL on the regression of pancreatic carcinoma in vivo, we have established a xenograft model using MIA PaCa-2 cells. The combined treatment regimen of 2-methoxyestradiol and TRAIL exerted significant inhibitory effect on the pancreatic tumor growth in athymic mice.

Materials and Methods

Cell lines. Human pancreatic cancer cell lines MIA PaCa-2, AsPC-1, and Hs766T were grown in RPMI supplemented with 10% fetal bovine serum and 50 μg/mL gentamicin at 37°C in a 5% CO2 humidified atmosphere.

Apoptosis assay. Cells (5 × 104) were seeded (in triplicate plates) in the growth medium and, the next day, were treated with 2-methoxyestradiol in the presence or absence of Jun kinase inhibitor (EMD Biosciences, San Diego, CA), caspase inhibitors, and death receptor ligands FasL/TRAIL (R&D Systems), in growth medium and, the next day, were treated with 2-methoxyestradiol in 100 mm/L of 25 mmol/L HEPES (pH 7.2), 142 mmol/L KCl, 5 mmol/L MgCl2, 1 mmol/L EGTA, 0.2% NP40, and protease inhibitors. After overnight incubation at 4°C, the immunocomplex was trapped in Protein A-Sepharose CL-4B beads (Amersham Biosciences) by incubating for 2 hours at 4°C. After washing, the kinase reaction for JNK was done by incubating the immunoprecipitated proteins in 25 mmol/L HEPES (pH 7.5), 25 mmol/L MgCl2, 25 mmol/L β-glycerophosphate, 1 mmol/L DTT, 0.1 mmol/L sodium orthovanadate, 10 μmol/L ATP, 1 μg GST-Jun (EMD Biosciences), and 10 μCi [γ-32P]ATP. Reactions were carried out at 30°C for 15 minutes. Kinase reactions were terminated by boiling with Laemmli’s sample buffer followed by SDS-PAGE and autoradiography (36, 37).

RPA. The status of several members of death receptor family in 2-methoxyestradiol-exposed pancreatic cancer cells was evaluated by RPA. RPA was done as described under the BD Riboquant multi-probe kit Instruction Manual (BD Biosciences). On the first day, 10 mg of total RNA from each sample were hybridized with 8 × 105 cpm of labeled probe template set hapo-3d in a final volume of 10 mL of hybridization buffer. Hybridization reactions were initiated in a heat block prewarmed to 90°C and allowed to cool down slowly to 56°C. The next day, the samples were treated with RNaseA/RNaseT1 for 45 minutes at 30°C followed by RNase K (10 mg) digestion for 15 minutes at 37°C. The samples were phenol/chloroform extracted, ethanol precipitated, and resuspended in 3 mL of loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromomophenol blue, 0.5 mmol/L EDTA, and 0.025% SDS). The RNase-protected fragments were analyzed on a 5% denaturing polyacrylamide gel (19:1 acrylamide/bis). Gels were transferred to filter paper, vacuum-dried, and exposed to X-ray film overnight. Films were processed in a Kodak film developer. The intensity of the signals was quantitated using a Phosphor-Imager system (Molecular Dynamics, Piscataway, NJ, Storm 860) and normalized using the housekeeping gene L32.

Cell surface Fas/DR4/DR5 analysis. MIA PaCa-2 cells were treated with 5 μmol/L 2-methoxyestradiol for 24 hours. The adherent cells were detached by non enzymatic cell dissociation buffer (Sigma). Both floating and adherent cells were washed with PBS and resuspended in ice-cold staining buffer (eBioscience, San Diego, CA). Cells (0.5 × 106) were incubated on ice for 15 minutes with 50 μg/mL γ-globulin (MP Biomedicals, Solon, OH). Subsequently, cells on ice were stained in the dark for 1 hour with phycoerythin-conjugated anti-Fas, anti-DR4, anti-DR5, or immunoglobulin G1 isotype control antibody (eBioscience) in accordance to the manufacturer’s instructions. Following staining, cells were washed thrice by staining buffer and analyzed by flow cytometry. A total of 10,000 events were monitored.

Treatment of cells with neutralizing antibodies to the death receptors. To determine whether 2-methoxyestradiol-mediated apoptosis occurs through the activation of death receptors, MIA PaCa-2 cells were pretreated with 800 ng/mL mouse anti-Fas neutralizing antibody, ZB-4 (Upstate Biotechnology, Lake Placid, NY) or 10 ng/mL neutralizing polyclonal goat antibody (R&D Systems) raised against extracellular domain of TRAIL-R1 (anti-DR4) for 3 hours before treating the cells with 2-methoxyestradiol. Following treatment with 2-methoxyestradiol for 24 hours, apoptosis was determined by DAPI staining as described above.

Tumor xenograft experiments. Athymic mice (6 weeks of age) were s.c. inoculated on the right flank with 2 × 106 human pancreatic cancer cells MIA PaCa-2 (38). After tumor formation, mice were randomized into four groups each containing five animals. These groups of mice either received recombinant TRAIL from R&D Systems (5 μg/kg body weight) or purified 2-methoxyestradiol (4). In the current report, we investigated whether activation of caspase-8 plays any role in the case of 2-methoxyestradiol-triggered apoptotic cell death in pancreatic carcinoma cells. Transient expression of dominant-negative caspase-8 (27) or dominant-negative FADD (28) can attenuate the effect of 2-methoxyestradiol-mediated cell death. In parallel with this observation, Z-IETD-FMK, a cell permeable irreversible inhibitor of caspase-8, can render significant protection against 2-methoxyestradiol-induced apoptosis. To understand the mechanism of caspase-8 activation, RNase protection assay (RPA) using multiprobe template (29) set indicates the up-regulation of Fas, a member of the death receptor family.

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Immunoblotting. Pancreatic cancer cells at 5 × 104 per 100-mm dish were seeded and treated 1 day later with specified concentrations of various agents. Cells were preincubated for 2 to 6 hours with 50 to 100 μmol/L Z-IETD-FMK or Z-LEHD-FMK (R&D Systems), 10 μmol/L JNK inhibitor II (EMD Biosciences) followed by 24-hours exposure of 5 μmol/L of 2-methoxyestradiol. For studies with death receptor ligands, cells were coincubated with 5 to 10 ng/mL TRAIL, (R&D Systems) and 5 μmol/L 2-methoxyestradiol for designated time periods. To investigate the effect of Fasl, cells were incubated with recombinant human Fasl (5-10 ng/mL) together with 10 μg/mL cross-linking antibody (mouse anti-6-histidine; R&D Systems). Following treatment, total cellular proteins were extracted (32–36). After normalization for total protein content, the resulting lysate was subjected to SDS-PAGE and blotted into nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Membranes were probed with the following antibodies: phospho-JNK, pro-caspase-8, pro-caspase-9 (Santa Cruz Biotechnology, Santa Cruz, CA), pro-caspase-3, FADD (BD Biosciences), cleaved caspase-3 (R&D Systems), Fasl (R&D Systems), and PARP (BD Biosciences). Immunodetection was accomplished by enhanced chemilu-
Levels of procaspase-3 or active caspase-3 in tumor tissues. Mice were anaesthetized, and tumors were harvested. Mined tumor tissues were homogenized using Tissue Tearor (Biospec Products, Inc., Bartlesville, OK) in lysis buffer containing 50 mmol/L HEPES (pH 7.2), 150 mmol/L NaCl, 0.2% NP40, 2 mmol/L EDTA, and 1% SDS followed by boiling for 10 minutes and centrifugation at 16 000 × g for 5 minutes at room temperature (39). The supernatant was subjected to immunoblotting with procaspase-3 antibody or cleaved caspase-3–specific antibody.

Results

Requirement of both caspase-8 and caspase-9 for 2-methoxyestradiol-mediated apoptosis of pancreatic carcinoma cells. Despite the evidence of Bid cleavage in 2-methoxyestradiol-induced apoptosis of MIA PaCa-2 cells (4), it was not clear which initiator caspase (caspase-8 or caspase-10) was involved. With the aim of determining the role of these caspases, MIA PaCa-2 cells were pretreated with cell permeable inhibitors of caspase-8 (Z-IETD-FMK) or caspase-10 (Z-AEVD-FMK) followed by 2-methoxyestradiol exposure. Interestingly, pretreatment of caspase-8 inhibitor but not caspase-10 inhibitor can significantly abrogate 2-methoxyestradiol-triggered cell death (Fig. 1A). Moreover, the specificity of activation of downstream caspases, such as caspase-9 or caspase-6, was further proven by employing specific inhibitors of caspase-6 and caspase-9. Prior addition of specific inhibitor of caspase-9 (Z-LEHD-FMK) can diminish 2-methoxyestradiol-induced cell death, whereas inhibitor of caspase-6 (Z-VEID-FMK) was without any effect (Fig. 1A). The Western blot with caspases-6 antibody also does not indicate any reduction in procaspase-6 level following 2-methoxyestradiol exposure (Fig. 1B).

To show whether caspase-8, caspase-9, or FADD are required, we quantitated apoptosis in cells transfected with pcDNA3 vector, dominant-negative caspase-8, dominant-negative FADD, and dominant-negative caspase-9, respectively. In contrast to empty vector, all these dominant-negative constructs inhibited 2-methoxyestradiol-induced apoptosis (Fig. 1C). To determine the kinetics of activation of caspase-8 and caspase-9, cells were treated with 2-methoxyestradiol for 8 to 24 hours time period, and the activity was assessed by the disappearance of the inactive forms procaspase-8 and procaspase-9 on Western blot. The two species of procaspases revealed are previously reported isoforms (40). Figure 1D shows that the activation of both procaspase-8 and procaspase-9 is necessary for 2-methoxyestradiol-induced apoptosis of MIA PaCa-2 cells as early as 16 hours. Cumulatively, investigation with caspase inhibitors and dominant-negative constructs hints us the simultaneous involvement of death receptor and mitochondrial events associated with 2-methoxyestradiol-triggered programmed cell death of pancreatic cancer cells.

**Figure 1.** Effect of caspase inhibitors/dominant-negative caspases on 2-methoxyestradiol (2-ME)–induced apoptosis of MIA PaCa-2 cells. A, inhibition of 2-methoxyestradiol-triggered cell death by both caspase-8 and caspase-9 inhibitors. Cells were preincubated for 2 hours with either 50 μmol/L Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), Z-VEID-FMK (caspase-6 inhibitor), or Z-AEVD-FMK (caspase-10 inhibitor) followed by a 24-hour exposure of 5 μmol/L 2-methoxyestradiol. Apoptotic nuclei were visualized by DAPI staining. Approximately 500 cells were scored from randomly chosen field for each category. Significant inhibition of 2-methoxyestradiol–induced apoptosis was noted in the presence of caspases-8 and caspases-9 inhibitors (P < 0.0005). B, effect of 2-methoxyestradiol on caspase-6 activation. Cells were treated with either vehicle solvent DMSO or 5 μmol/L 2-methoxyestradiol for 24 hours. Total proteins isolated from control and treated cells were subjected to Western blot with antibody against procaspase-6. C, attenuation of 2-methoxyestradiol–induced apoptosis by dominant-negative constructs of caspase-9 (DN-Casp9; P < 0.0005), caspase-8 (DN-Casp8; P < 0.001), and FADD (DN-FADD; P < 0.0005). Cells were transfected with either vector (pcDNAs) or the indicated constructs by calcium phosphate coprecipitation method. Twenty-four hours after transfection, cell culture medium was replaced with the medium containing 5 μmol/L 2-methoxyestradiol. Following 24 hours of 2-methoxyestradiol treatment, cells were harvested and stained with DAPI for scoring apoptotic nuclei as described in (A). D, kinetics of 2-methoxyestradiol–induced procaspase-8 and procaspase-9 activation. MIA PaCa-2 cells were challenged with 5 μmol/L 2-methoxyestradiol for 8 to 24 hours, and, subsequently, cellular extract was immunoblotted with procaspase-8 and procaspase-9 antibodies.

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Caspase-8 acts upstream of caspase-9 and caspase-3. In the case of 2-methoxyestradiol-mediated apoptosis of Ewing sarcoma cells, previous report indicated that activation of caspase-8 could occur by caspase-9-mediated caspase-3 activation (15). Here, we were interested to test the sequence of activation of these caspases. MIA PaCa-2 cells were pretreated with 50 μmol/L, Z-IETD-FMK, a specific inhibitor of caspase-8, and then exposed to 5 μmol/L 2-methoxyestradiol for a 24-hour time period. The analysis of the status of procaspase-3 by immunoblotting reveals the ability of caspase-8 inhibitor to attenuate the disappearance of procaspase-3 caused by 2-methoxyestradiol alone (Fig. 2, middle, lane 3 versus lane 2). Concurrently, the regressing apoptosis was noted under identical condition as evident from lesser extent of PARP cleavage. Furthermore, caspase-9 inhibitor can block both 2-methoxyestradiol-induced caspase-3 activation and PARP cleavage (lanes 4 and 5). Because our studies using caspase inhibitors indicate that caspase-8 regulates caspase-3 activity in 2-methoxyestradiol-mediated demise of pancreatic carcinoma cells, the possibility of caspase-8 acting downstream of caspase-9 is unlikely. Previous reports (41, 42) show that caspase-9 regulates caspase-8 through the activation of caspase-3. In an attempt to corroborate the inhibitor studies presented in Fig. 2A, we further analyzed the activation status of caspase-8, caspase-9, and caspase-3 in vector- and dominant-negative caspase-9–transfected MIA PaCa-2 cells. As presented in Fig. 2B, the activation of caspase-9, caspase-3, and caspase-8 are evident in 2-methoxyestradiol-challenged pcDNA3 vector-transfected cells (lane 2). However, transfection of dominant-negative caspase-9 can significantly abrogate activation of both caspase-9 and caspase-3 without any effect on caspase-8 (lane 3). Our scanning data reveal the decrease in the level of procaspase-8 (≤2-fold) in both lanes 2 and 3 compared with lane 1. In all, our findings conform to the notion that caspase-8 is upstream of caspase-9 activation in 2-methoxyestradiol-treated pancreatic cancer cells.

2-Methoxyestradiol exposure induces up-regulation of death receptors in pancreatic cancer cells. The significant inhibition of 2-methoxyestradiol-triggered apoptosis by transfection of dominant-negative construct of FADD (Fig. 1C) led us to examine whether any member of death receptor family plays any role in our model. Several death receptors mediate apoptosis by recruiting FADD to the oligomerized death receptor complex, where FADD facilitates the binding and activation of procaspase-8 or procaspase-10. On this basis, we undertook the approach of RPA using multiprobe template set. The multiprobe RPA systems include a series of templates of distinct length, and each represents a sequence in a distinct mRNA species. The templates are assembled into biologically relevant sets. In our case, the set contained specific probes for a variety of genes coding for death receptor family members (DR3, DR4, DR5, Fas, Fasl, etc.), caspase-8, and internal controls, such as GAPDH and L32. Following 24 hours of treatment with 5 μmol/L 2-methoxyestradiol, RPA indicates up-regulation of Fas in MIA PaCa-2 cells (Fig. 3A, lane 2) in contrast to 2-methoxyestradiol-exposed Hs766T cells. Of note, metastatic pancreatic cancer cells Hs766T do not undergo apoptosis following 2-methoxyestradiol exposure (4). Quantitation of death-related genes revealed in RPA by ImageQuant software indicated a 2-fold increase in Fas mRNA in 2-methoxyestradiol-exposed MIA PaCa-2 cells (Fig. 3A) when counts were normalized against L32. Figure 3B shows results obtained from experiments where we investigated the effect of 2-methoxyestradiol on cell surface Fas, DR4, and DR5 expression using flow cytometry. 2-Methoxyestradiol substantially increased Fas expression (2.2-fold), but little effect was observed in DR5 level. However, 2-methoxyestradiol induced a 1.3-fold increase in DR4 level. In the case of synthetic triterpenoid–mediated apoptosis of breast cancer cells, similar moderate increase of TRAIL receptors was reported (43). Although both RPA and cell surface receptor analysis could detect 2-methoxyestradiol-induced Fas up-regulation, a modest increase in surface DR4 was evident only in the flow cytometry analysis of 2-methoxyestradiol-treated cells. The failure to detect an increase in cellular DR4 RNA level by RPA might be attributed to 2-methoxyestradiol-induced possible redistribution of DR4. This redistribution might traffic more receptors to the cell surface without detectable alteration of total DR4 level (43). 2-Methoxyestradiol-induced up-regulation of Fas.

Figure 2. Sequence of activation of caspases in 2-methoxyestradiol (2-ME)-challenged MIA PaCa-2 cells. A, effect of caspase-9/caspase-8 inhibitors on the activation of procaspase-3. Cells were preincubated with 50 to 100 μmol/L caspase-8 or caspase-9 inhibitors for 2 hours followed by incubation with 5 μmol/L 2-methoxyestradiol for another 24 hours. Subsequently, cell lysate was subjected to Western blot with PARP and caspase-3 antibodies. Lane 1, control; lane 2, 5 μmol/L 2-methoxyestradiol; lane 3, 50 μmol/L caspase-8 inhibitor followed by 5 μmol/L 2-methoxyestradiol; lane 4, 50 μmol/L caspase-9 inhibitor followed by 5 μmol/L 2-methoxyestradiol; lane 5, 100 μmol/L caspase-9 inhibitor followed by 5 μmol/L 2-methoxyestradiol. B, effect of transfection of dominant-negative construct of caspase-9 (DN-caspase-9) on activation of procaspase-8 and procaspase-3. Twenty-four hours after transfection, transfected cells were either treated with vehicle solvent (DMSO) or 5 μmol/L 2-methoxyestradiol for 16 hours. Total cellular extract was normalized for total protein content and analyzed by immunoblotting using procaspase-9, procaspase-3, and procaspase-8 antibodies. Furthermore, the bands of procaspase-8 and tubulin in each lane were scanned, and the signals were quantitated using Scion Image for Windows software (Scion Corp., Frederick, MD). The numbers at the bottom represent the value of ratio between signal intensities of procaspase-8 and tubulin.
RNA in MIA PaCa-2 cells was further confirmed by Western blot with Fas monoclonal antibody (Fig. 3C). However, we were unable to show the elevated level of caspase-8 protein in the presence of its specific inhibitor despite moderate increase in RNA level by RPA (Fig. 3A). Notably, caspase-8 inhibitor pretreatment attenuated the activation of caspase-8 (data not shown). Perhaps, the negative translational control for caspase-8 expression might preclude the up-regulation of caspase-8 protein despite some elevation at RNA level in 2-methoxyestradiol-exposed cells.

Because death receptors, Fas, or DR4 recruit adapter molecule FADD for rendering apoptotic signaling, we tested whether 2-methoxyestradiol can elevate the level of FADD. As shown in Fig. 3D, along with Fas, FADD elevation was also noted in 2-methoxyestradiol-exposed MIA PaCa-2 cells. Possibly, 2-methoxyestradiol-mediated Fas/DR4 up-regulation augments apoptotic signaling by recruiting FADD to form death-inducing signaling complex (DISC).

Jun kinase acts upstream of Fas up-regulation. The mechanism by which 2-methoxyestradiol can alter cell surface expression of death receptors is not known. It might involve the disruption of intracellular redox balance, leading to JNK and subsequent up-regulation of members of death receptor family as observed earlier (43–45). Primarily, we assessed the status of JNK activity by immunoblotting of cellular extract prepared from 2-methoxyestradiol-exposed MIA PaCa-2 cells with monoclonal FADD antibody. Lane 1, control; lane 2, 5 μmol/L 2-methoxyestradiol treatment for 16 hours.

Figure 3. Effect of 2-methoxyestradiol (2-ME) on the expression of death receptor–related genes in pancreatic cancer. A, quantitation of death-related genes revealed in RPA. MIA PaCa-2 and Hs766T cells were treated with or without 5 μmol/L 2-methoxyestradiol for a period of 24 hours. Total RNA was used in RPA using hAPO-3d probe (BD Biosciences). L32 and GAPDH are shown as housekeeping genes. Lanes 1 and 3, control; lanes 2 and 4, 2-methoxyestradiol treated. Lanes 1 and 2, MIA PaCa-2 cells; lanes 3 and 4, Hs766T cells (inset). ImageQuant software was used to scan each band. Counts were normalized against L32. B, dot plot of cell surface expression of death receptors. Cell surface Fas, DR4, and DR5 expression was measured by flow cytometry on 2-methoxyestradiol treated (as above) MIA PaCa-2 cells using phycoerythrin (PE)–conjugated anti-Fas, anti-DR4, anti-DR5, and IgG1 isotype control antibodies. Note that 2-methoxyestradiol exposure increases cell surface Fas and DR4 by 2.2-fold (P < 0.002) and 1.3-fold (P < 0.05), respectively. C, elevated level of Fas protein following 2-methoxyestradiol exposure. MIA PaCa-2 cells were challenged with 5 μmol/L 2-methoxyestradiol for 8 to 24 hours. Western blot was carried out using Fas monoclonal antibody. Lane 1, control; lanes 2-4, 2-methoxyestradiol exposure for 8, 16, and 24 hours, respectively. D, effect of 2-methoxyestradiol on FADD level. FADD protein was detected by immunoblotting of cellular extract prepared from 2-methoxyestradiol-exposed MIA PaCa-2 cells with monoclonal FADD antibody. Lane 1, control; lane 2, 5 μmol/L 2-methoxyestradiol treatment for 16 hours.
the effect of JNK inhibitor II on cell death. As shown in Fig. 4B and D, either preincubation with 10 μmol/L JNK inhibitor II or transfection with dominant-negative JNK1 rendered MIA PaCa-2 cells refractory to 2-methoxyestradiol-mediated apoptosis. Interestingly, pretreatment of cells with JNK inhibitor II or exogenous expression of dominant-negative JNK1 also prevents 2-methoxyestradiol-induced up-regulation of Fas (Fig. 4C and D). All together, our investigation suggests that JNK activation acts upstream of death receptor up-regulation in 2-methoxyestradiol-induced cell death pathway. To assess whether oxidative stress precedes JNK activation, we treated cells with a well-known antioxidant N-acetylcysteine (NAC) before 2-methoxyestradiol exposure. When MIA PaCa-2 cells are exposed to 5 μmol/L 2-methoxyestradiol alone, JNK is phosphorylated as indicated by Western blot with phospho-JNK-specific antibody (Fig. 4E, lane 1 versus lane 6). Primarily, this confirms our results on 2-methoxyestradiol-mediated JNK phosphorylation obtained by immunocomplex kinase assay (Fig. 4A). However, 2-methoxyestradiol-mediated phosphorylation of JNK can be diminished if cells are pretreated with 5 mmol/L NAC for 2 hours (Fig. 4E, top, lane 5). Notably, pretreatment with 1 mmol/L NAC has little effect on the prevention of JNK1 phosphorylation. Correlatively, 2-methoxyestradiol-induced PARP cleavage is also blocked in cells pretreated with 5 mmol/L NAC (Fig. 4E, middle, lane 5). All together, data presented here reveal the apparent requirement of 2-methoxyestradiol-generated oxidative stress to activate JNK.

2-Methoxyestradiol sensitizes pancreatic cancer cells to Fas or TRAIL. RPA approach and cell surface death receptor analysis indicates significant up-regulation of a member of TNF receptor family, such as Fas (Fig. 3), along with a moderate increase in one of the TRAIL receptors, DR4. However, little increase in DR5 was noted following exposure to 2-methoxyestradiol (Fig. 3B). Moreover, the neutralizing antibodies against these receptors (Fas or DR4) can significantly inhibit 2-methoxyestradiol-triggered cell death of MIA PaCa-2 cells (Fig. 5A). Fifty-five percent of 2-methoxyestradiol-induced apoptosis is blocked if the cells are pretreated with Fas-neutralizing antibody. However, neutralizing antibody against DR4 can attenuate this apoptosis nearly by 35% (Fig. 5A). Accordingly, we tested the effects of death receptor ligands Fasl or TRAIL, when used in combination with

Figure 4. Inactivation of Jun kinase can attenuate 2-methoxyestradiol (2-ME)–induced Fas up-regulation and apoptosis. A, immunocomplex kinase assay in 2-methoxyestradiol-exposed pancreatic cancer cells. JNK was immunoprecipitated from control cells and cells exposed to 5 μmol/L 2-methoxyestradiol for 24 hours using antibody against JNK. Immunocomplexes were assessed for their ability to phosphorylate GST-c-Jun (1-79 amino acids) as described in Materials and Methods. Lanes 1 and 3, GST-Jun phosphorylation in untreated cells; lanes 2 and 4, GST-Jun phosphorylation in cells treated with 5 μmol/L 2-methoxyestradiol for 24 hours. Lanes 1 and 2, MIA PaCa-2 cells; lanes 3 and 4, Hs 766T cells. B and C, effect of JNK inhibitor II (SP60025) on 2-methoxyestradiol-induced apoptosis and Fas up-regulation. MIA PaCa-2 cells were pretreated with 10 μmol/L JNK inhibitor II for 6 hours followed by 24-hour treatment with 5 μmol/L 2-methoxyestradiol. The cells were either stained with DAPI (B) to assess apoptosis by fluorescence microscopy or subjected to Western blot analysis (C) with Fas antibody. Inhibition of 2-methoxyestradiol-induced apoptosis by JNK inhibitor II was statistically significant (P < 0.0005). D, transient transfection of dominant-negative JNK1 (DN-JNK1) inhibits 2-methoxyestradiol-induced apoptosis (P < 0.0005) and Fas up-regulation. pcDNA3 vector and dominant-negative JNK1–transfected MIA PaCa-2 cells were exposed to 5 μmol/L 2-methoxyestradiol for 24 hours and analyzed by DAPI staining or immunoblotting with Fas antibody. Lane 1, pcDNA3 (no 2-methoxyestradiol); lane 2, pcDNA3 (2-methoxyestradiol treated); lane 3, DNJNK1 (2-methoxyestradiol treated). E, antioxidant NAC prevents 2-methoxyestradiol-mediated JNK activation and PARP cleavage in MIA PaCa-2 cells. Cells were treated with 1 or 5 mmol/L NAC for 2 hours before 2-methoxyestradiol exposure. Cell-free extract was analyzed for phospho-JNK level and PARP cleavage. Lane 1, 2-methoxyestradiol alone; lane 2, 1 mmol/L NAC; lane 3, 1 mmol/L NAC plus 2-methoxyestradiol; lane 4, 5 mmol/L NAC; lane 5, 5 mmol/L NAC plus 2-methoxyestradiol; lane 6, control.
2-methoxyestradiol in MIA PaCa-2 cells. As evident in Fig. 5B and C, both FasL and TRAIL potentiate 2-methoxyestradiol-induced PARP cleavage and procaspase-3 activation, which are hallmarks of apoptosis (20, 32). We also tested the synergistic effects of FasL/TRAIL and 2-methoxyestradiol in other pancreatic cancer cells. Like 2-methoxyestradiol, neither TRAIL nor FasL alone induced significant apoptosis in cultures of AsPC-1 (Fig. 5D). In contrast, the exposure to 2-methoxyestradiol and TRAIL or FasL can significantly sensitize them to cell death (Fig. 5D). In addition, combination treatment enhanced PARP degradation in other pancreatic cancer cells, such as PANC-1 (data not shown).

2-Methoxyestradiol cooperates with TRAIL to reduce tumor burden in vivo. To determine whether 2-methoxyestradiol in combination with TRAIL inhibits tumor growth in vivo, we implanted MIA PaCa-2 cells into the subcutis of athymic mice. s.c. tumor-bearing mice were treated as described in Materials and Methods. Combined treatment with 2-methoxyestradiol and TRAIL significantly inhibited tumor growth compared with either agent alone (Fig. 6A). Further analysis of tumor samples revealed that the combination treatment of 2-methoxyestradiol and TRAIL augmented apoptosis in mice compared with 2-methoxyestradiol or TRAIL alone. As shown in Fig. 6B (lanes 3 and 4), significant disappearance of procaspase-3 level was evident in tumor tissues harvested after the combination treatment of 2-methoxyestradiol and TRAIL. To show that tumors of mice treated with 2-methoxyestradiol and TRAIL indeed display caspase-3 activation, we analyzed tumor lysate (Fig. 6B, samples 2, 4, 6, and 8) to detect cleaved caspase-3 (19-kDa fragment) by immunoblotting using antibody specific for active caspase-3. Tumor treated with 2-methoxyestradiol and TRAIL exhibits significant cleavage of caspase-3 (Fig. 6C, lane 2). This data corroborate the greatest extent of disappearance of procaspase-3 as observed in the same sample (Fig. 6B, lane 4).

![Figure 5.](image-url)

**Figure 5.** Combinatorial effect of 2-methoxyestradiol (2-ME) and death receptor ligands on death of pancreatic cancer cells. A, effects of neutralizing antibodies to Fas and DR4 on 2-methoxyestradiol-induced apoptosis. MIA PaCa-2 cells were pretreated with neutralizing anti-Fas, anti-DR4 antibodies for 3 hours before treatment with 5 μmol/L 2-methoxyestradiol for 24 hours. Normal mouse and goat IgGs were also used as control isotype antibodies. Apoptosis was determined by DAPI staining. Note that the extent of apoptosis induced by 2-methoxyestradiol alone was considered to be 100%. Inhibition of apoptosis by anti-Fas antibody (P < 0.005) and anti-DR4 antibody (P < 0.005) was statistically significant when compared with normal mouse IgG and Goat IgG antibodies, respectively. B-D, synergistic effect of 2-methoxyestradiol and TRAIL/FasL on pancreatic cancer cell death in vitro. MIA PaCa-2 cells were exposed to either 5 μmol/L 2-methoxyestradiol or 5 ng/mL recombinant human TRAIL or 5 μmol/L 2-methoxyestradiol and 5 ng/mL TRAIL together for 16 hours (B). Cell lysate was subjected to Western blot analysis using monoclonal antibody against PARP and procaspase-3 antibody. C, MIA PaCa-2 cells were treated for 24 hours with 5 μmol/L 2-methoxyestradiol in the presence or absence of recombinant human FasL (5 ng/mL) together with 10 μg/mL cross-linking antibody (mouse anti-6-histidine). Following designated period of exposure, apoptosis was determined by PARP cleavage as described previously in Fig. 2. AsPC-1 cells were treated as described in (C), except 10 ng/mL TRAIL/FasL was used and apoptosis was determined by nuclear staining (D) as described previously in Fig. 1. FasL/TRAIL in combination with 2-methoxyestradiol enhanced apoptosis compared with 2-methoxyestradiol alone (P < 0.0001).
Discussion

Pancreatic cancer is one of the major causes of all cancer morbidity in the United States (4, 36). The aberrant biological and biochemical properties of the pancreatic cancer cell acquired by several activating mutations might contribute to resistance to chemotherapeutics. The developing strategy, such as targeting apoptotic signaling machinery in pancreatic cancer, is thought to be a promising alternative. The data presented in this report indicate that 2-methoxyestradiol, an estrogen metabolite, sensitizes pancreatic cancer cells to death receptor ligand FasL, or TRAIL-mediated apoptosis possibly by up-regulation of death receptor Fas as well as to some extent DR4 (Fig. 3). Earlier report shows a close link between death receptor signaling and stress response kinase JNK (45). In several human cancer cells, it was reported that JNK activation and death receptor activation could synergistically act to induce cell death (45, 46). However, the mechanism by which 2-methoxyestradiol can alter the expression of cell surface death receptors Fas or DR4 in Mia PaCa-2 cells is obscure. One possibility arises from the rapid activation of JNK by 2-methoxyestradiol, which might lead to increased activator protein (AP-1) expression as reported earlier (45). By AP-1-mediated transcriptional regulation mechanism (47, 48), Fas expression might be elevated in 2-methoxyestradiol-exposed pancreatic cancer cells. Thus, in our model, JNK pathway perhaps by AP-1-mediated transcriptional regulation of death receptor contributes to cell death. Of note, an AP-1 binding site was located on the promoter of death receptor genes, including Fas (47, 48).

The ability of 2-methoxyestradiol to trigger enhanced production of reactive oxygen species (ROS) in cancer cells is known (12, 15). ROS, comprising of hydroxyl radicals, superoxide anion, or hydrogen peroxide, are produced by external stresses (36, 49). The cellular defense mechanism to ROS production consists of antioxidant scavengers or antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (36, 49). 2-Methoxyestradiol is known to inhibit superoxide dismutase enzyme activity (12) and thus might generate excessive ROS to perturb the oxidation-reduction (redox) reactions. The perturbation in redox balance plays a critical role for inducing apoptosis in response to chemotherapeutics in cancer cells (36, 44, 46, 50). Because antioxidant NAC at 5 mmol/L concentration can inhibit the activation of JNK by 2-methoxyestradiol as well as apoptosis (Fig. 4E), it is quite possible that early generation of ROS by the inhibition of antioxidant enzyme superoxide dismutase might escalate JNK activation in pancreatic cancer cells (Fig. 7). Although we have not measured ROS production, the inability of 1 mmol/L NAC to prevent 2-methoxyestradiol-induced JNK phosphorylation/apoptosis might be due to lack of inhibition of ROS generation.

Our systematic studies using specific inhibitors of caspases as well as dominant-negative constructs of JNK1, FADD, caspase-8, and caspase-9 dissect the molecular mechanism of 2-methoxyestradiol-mediated cell death in pancreatic cancer. As depicted in Fig. 7, 2-methoxyestradiol-induced activation of death receptor signaling followed by caspase-8 activation converges with mitochondrial machinery of apoptosis. To the best of our knowledge, we report for the first time the hierarchy of molecular events involving death receptor Fas, which is associated with 2-methoxyestradiol-mediated cell death in pancreatic cancer. Interestingly, 2-methoxyestradiol inhibits angiogenesis in bovine pulmonary artery endothelial cells by inducing apoptosis. 2-Methoxyestradiol causes endothelial cell apoptosis possibly by activation of JNK signaling and up-regulation of the Fas death receptor (10), which is concordant with our findings reported here (Figs. 3 and 4).

Fas (CD95) is one of the best characterized members of the TNF superfamily of receptors (51). Upon binding with Fasl, activated Fas receptors signal the apoptotic response by recruiting FADD to form the DISC. In pancreatic cancer cells, the concerted effect of 2-methoxyestradiol-induced elevated levels of Fas/DR4 as well as the adapter protein FADD might play a pivotal role in initiating apoptotic cascade. Interestingly, we observed two closely migrating forms of FADD due to 2-methoxyestradiol exposure (Fig. 3D), and both forms were increased. The slower mobility form might be phosphorylated FADD as reported earlier (52). Phosphorylated FADD has been shown to play an essential role in the amplification of chemotherapy-induced apoptosis of prostate cancer cells (52). In pancreatic cancer, the comprehension of the role of elevated forms of FADD in 2-methoxyestradiol-induced apoptosis warrants further investigation.

Because TRAIL/Apo2L/ TNFSF10 can evoke cell death in many types of cancer cells without affecting normal cells (53), the interest
in employment of this agent as therapeutic for cancer is increasing. The combination regimen of 2-methoxyestradiol and TRAIL was significantly effective to sensitize these pancreatic cancer cells to killing. Not only TRAIL but also TNF-α, another ligand of death receptor family, potentiates 2-methoxyestradiol-triggered cell death of Ewing sarcoma cells (54). Apparently, the death receptor ligand, such as FasL or TRAIL, augments 2-methoxyestradiol-triggered apoptosis.

To summarize, this is the first evidence that 2-methoxyestradiol potently sensitizes death receptors in pancreatic carcinoma. The death receptor ligands and 2-methoxyestradiol may act in concert to enhance pancreatic carcinoma cytotoxicity through extrinsic and intrinsic apoptotic pathways. These two pathways collectively amplify apoptotic events involving mitochondria, leading to activation of caspase-3, the central executioner of the cell death signaling pathways. Our report on 2-methoxyestradiol-induced death receptor signaling in pancreatic carcinoma conforms to earlier observation reported in the case of breast cancer (14). Recently, 2-methoxyestradiol was reported not to exert any effect on the up-regulation of DR5 in human glioma cell lines (55). The difference in the observation could be due to cell line specificity. However, the inhibition of 2-methoxyestradiol-induced apoptosis by dominant-negative constructs of FADD in both breast cancer (14) and pancreatic cancer (Fig. 1) argues in favor of the implication of the death receptor pathway.

Finally, this report provides the first preclinical in vivo data on the efficacy of an angiogenesis inhibitor 2-methoxyestradiol and death receptor ligand TRAIL with respect to suppression of heterotopic pancreatic tumor growth in mice. The synergistic effect of 2-methoxyestradiol and TRAIL was clearly evident in TRAIL-sensitive MIA PaCa-2 cells (56) in vitro and in vivo. Moreover, 2-methoxyestradiol can definitely sensitize TRAIL-resistant pancreatic cancer cells, such as A549 (Fig. 5) and lung cancer cells (17). Thus, it is quite possible that 2-methoxyestradiol might inhibit angiogenesis and augment apoptotic signaling in vivo to reduce tumor burden in our model. However, the potential therapeutic advantage of this combination treatment needs further exploration in other pancreatic tumor xenograft models.

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