

2-Methoxyestradiol, an Endogenous Mammalian Metabolite, Radiosensitizes Colon Carcinoma Cells through c-Jun NH₂-Terminal Kinase Activation

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Abstract Purpose: 2-Methoxyestradiol (2ME), an estrogen metabolite, induces apoptosis in various cell types. We investigated whether 2ME pretreatment can radiosensitize colon adenocarcinoma cells.

Experimental Design: Radiosensitizing effects of 2ME were evaluated by cell death, clonogenic assay, nuclear fragmentation, and tumor progression of xenografts. Ionizing radiation – induced DNA damage was evaluated by histone H2AX phosphorylation and its foci. The c-Jun NH₂-terminal kinase (JNK) activation was evaluated by anti-phosphorylated JNK antibody and inhibited by the JNK-specific inhibitor SP600125 or dominant-negative SEK1 expression.

Results: Clonogenic assays revealed that 2ME, but not estradiol, radiosensitized three colon carcinoma cells, DLD-1, HCT-8, and HCT-15, and strongly suppressed tumor progression of DLD-1 xenografts. Gene transfer – mediated Bcl-xL overexpression largely abolished both augmented apoptosis and reduced survival fractions. Pretreatment with 2ME enhanced H2AX phosphorylation, its foci, and phosphorylation of ATM kinase and delayed re-entry of cell cycle progression after ionizing radiation. Augmentation of both radiosensitivity and H2AX phosphorylation was substantially reduced by SP600125 or overexpression of a dominant-negative mutant SEK1.

Conclusion: 2ME radiosensitized colon carcinoma cells through enhanced DNA damage via JNK activation, thereby representing a novel radiosensitizing therapy against colon cancer.

2-Methoxyestradiol (2ME), which does not bind to the estrogen receptor, is a physiologic metabolite of the endogenous estrogen 17 β -estradiol (E₂; refs. 1, 2). E₂ is metabolically hydroxylated by NADPH-dependent cytochrome P450 and then O-methylated by catechol-O-methyltransferase to 2ME (3). 2ME at nonphysiologic concentrations induces apoptosis in various cancer cell types but is relatively nontoxic to normal cells (2, 4–6). This implies that 2ME induces a less adverse effect and thus might be clinically useful against a broad range of malignant cells.

Although 2ME and its derivatives are supposed to be clinically relevant compounds, the 2ME-mediated signaling cascades responsible for their anticancer activities remain unclear. Because affinity of 2ME to the estrogen receptors is minimal (2), estrogen receptor-mediated signals are unlikely to be involved in the proapoptotic activities. However, 2ME has a wide range of actions possibly associated with its proapoptotic activity. First, 2ME induces accumulation of reactive oxygen species (ROS) due to inhibition of superoxide dismutase (7–9). Second, 2ME activates hypoxia-inducible factor-1 α and c-Jun NH₂-terminal kinase (JNK) pathways (10–12). Third, 2ME-induced oxidative injury inactivates phosphatidylinositol 3-kinase and Akt, both of which play a critical role in survival signals (13). Fourth, 2ME binds to the colchicine site of tubulin and inhibits its polymerization, interferes with spindle dynamics during mitosis, and eventually leads to apoptosis (14). Fifth, 2ME up-regulates death receptor expression (15). Of these mechanisms, it remains uncertain which are involved in the proapoptotic activity of 2ME.

Ionizing radiation (IR) strongly induces growth arrest and/or apoptosis in a variety of cancer cells (16). However, sensitivity to IR differs markedly and gastrointestinal carcinoma cells are relatively resistant; therefore, radiation is not commonly chosen for therapy of primary gastrointestinal lesions. Nevertheless, many attempts have been made to evaluate radiation therapy in the management of locally advanced gastrointestinal cancers, and many scientists are striving to find novel and highly potent radiosensitizing agents. Indeed, several *in vitro*

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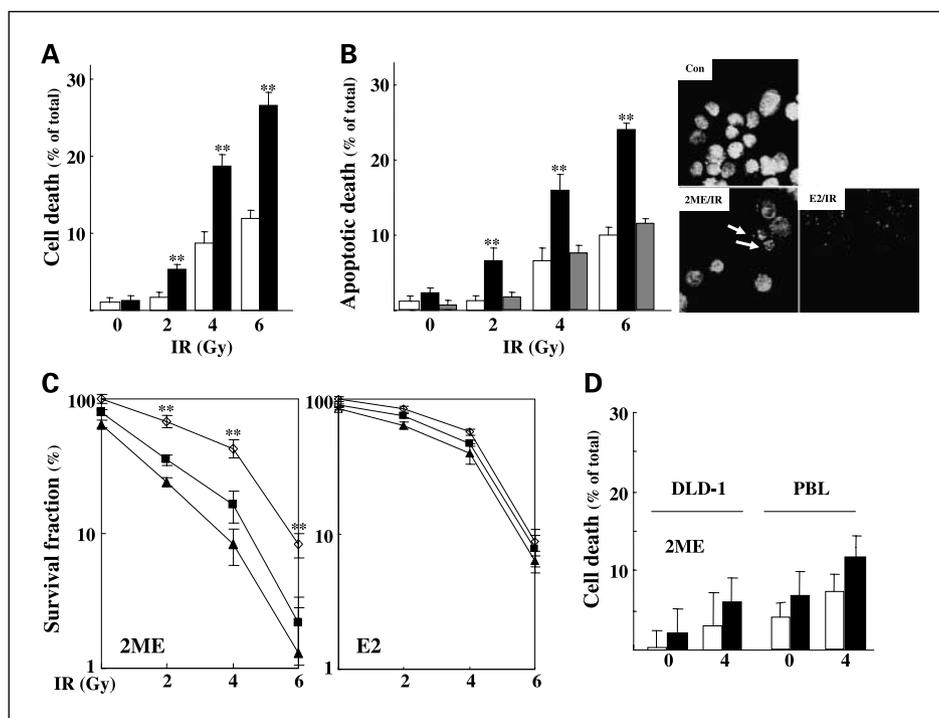
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Fig. 1. Effects of 2ME in combination with IR in DLD-1 cells. **A**, trypan blue exclusion assay. Cell death 48 hours after IR at the indicated doses followed by preincubation with (black columns) or without 2 $\mu\text{mol/L}$ 2ME for 12 hours. **B**, DAPI staining. Cells were pretreated with 2 $\mu\text{mol/L}$ 2ME (black columns) or E_2 (gray columns) for 12 hours, irradiated at the indicated doses, cultured without 2ME or E_2 for 48 hours, and stained with DAPI. Right, representative data. **C**, clonogenic assay. Cells (3,000 per dish) were cultured without or with 2ME or E_2 (■, 1 $\mu\text{mol/L}$; ▲, 2 $\mu\text{mol/L}$) for 12 hours, irradiated at the indicated doses, and cultured for 9 days without each agent. Colony numbers (>25 cells) were counted. Columns and points, mean of three separate experiments; bars, SD (A-C). **D**, trypan blue exclusion assay. Cells were exposed to IR (4Gy) and then treated with 2 $\mu\text{mol/L}$ 2ME (closed columns) for 12 hours. Cell death was evaluated 48 hours after IR. As a control study, normal lymphocytes (PBL) were treated as in (A), and cell death was evaluated 48 hours after IR. Columns, mean of three separate experiments; bars, SD. **, $P < 0.01$ compared with IR alone.



and *in vivo* studies show that inhibition of epidermal growth factor receptor, Ras, phosphatidylinositol 3-kinase, and Akt radiosensitizes cancer cell lines (17), and histone deacetylase inhibitors also strongly radiosensitize cancer cells (18, 19). We believe these agents have promising clinical potential and that radiation therapy will become potentially valuable, even for primary gastrointestinal carcinomas, if an effective safe radiosensitizer is identified.

We here addressed whether 2ME could enhance the radiosensitivity of colon carcinoma cells and showed that 2ME, but not E_2 , radiosensitized three different colon carcinoma cells and inhibited the tumor progression of DLD-1 xenografts. We also showed that 2ME increases IR-induced DNA damage and this activity depends on 2ME-mediated JNK activation. Because 2ME is a natural metabolite of estrogen and its preradiation use requires much smaller doses compared with its independent use, our findings outline a new concept for its use as a unique radiosensitizer.

Materials and Methods

Cell culture and IR exposure. Human colon DLD-1, HCT-8, and HCT-15 cancer cells, obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), were grown in RPMI 1640 supplemented with 10% FCS. Cells were exposed at room temperature to IR using 150 kV X-ray equipment (M-150WE, Softex, Tokyo, Japan), which can provide a dose rate of 110 cGy/min. To evaluate viability, cells were mixed with the same volume of 0.4% trypan blue solution and immediately examined to determine whether they could exclude the dye. For 4',6-diamidino-2-phenylindole (DAPI) staining assay, both floating and adherent cells were mixed, fixed in 70% ethanol for 10 minutes, and stained with DAPI (0.5 $\mu\text{g/mL}$ PBS) for 10 minutes, and chromatin fluorescence was observed under UV light using a confocal microscope (R2100AG2, Bio-Rad, Melville, NY).

Reagents. 2ME, E_2 , a JNK-specific inhibitor SP600125, and a specific p38 inhibitor SB202190 were obtained from Sigma (St. Louis,

MO). Antibodies against Bcl-xL (PharMingen, San Diego, CA); HA (12CA5, Roche Molecular Biochemicals, Indianapolis, IN); Bax, JNK, ATM, and 14-3-3 (Santa Cruz Biotechnology, Santa Cruz, CA); phosphorylated H2AX (Upstate Biotechnical); and BAD, BID, phosphorylated JNK, and phosphorylated ATM (Cell Signaling Technology, Beverly, MA) were used for Western blots or confocal microscopic analysis.

Tumor xenografts in nude mice and *in vivo* combination therapy. DLD-1 cells (2×10^6) were injected s.c. in the back of 4- to 5-week-old athymic nude mice. Tumor volumes were determined from caliper measurements of tumor length (L) and width (W) according to the formula $(L \times W^2) / 2$. When tumor volumes reached a minimal size of $40 \pm 10 \text{ mm}^3$ (approximately 14 days after cell injection), mice ($n = 6$, each group) were given a locoregional applied body dose of 4 Gy (two or three fractions weekly) using 120 kVp X-ray equipment (6 mA). 2ME (40 μL , 2 mmol/L) was intratumorally injected twice weekly 12 hours before irradiation. Tumor sizes were measured weekly until sacrifice, and tumor weight was measured at 3 weeks after treatment began. Total cell lysates of tumors were analyzed by Western blots. Tumors were also fixed in neutral-buffered formalin, embedded in paraffin, sectioned (5 μm), and stained with H&E for histologic analysis. Our institute approved these animal protocols.

Transfection. The human Bcl-xL full-length cDNA pcDNA3, constitutively active JNK (pEF-Flag-MKK7 β -JNK1), or dominant-negative MKK4 (pMT-HA-SEK1) expression vector was transfected into DLD-1 cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). The Bcl-xL pcDNA3 and pMT-HA-SEK1 are kindly provided from Dr. Yoshihide Tsujimoto (Osaka University, Osaka, Japan) and Dr. Mutsuhiro Takekawa (Tokyo University, Tokyo, Japan), respectively. The efficiency of transcription was achieved at 30% to 40% for DLD-1 cells, which was evaluated by transfection with pEGFP C3 expression vector (Invitrogen). At 12 hours after transfection, DLD-1 cells were replated for clonogenic assay or to evaluate expression of Bcl-xL or JNK and its phosphorylation.

Cell cycle analysis. Cells (1×10^6) were incubated with 0.1% Triton X-100 for 10 minutes, washed, further incubated with 25 $\mu\text{g/mL}$ propidium iodide, and 1 $\mu\text{g/mL}$ RNase A for 15 minutes and analyzed for cellular fluorescence by FACScan flow cytometry (Becton Dickinson, Mountain View, CA) using CellQuest Software.

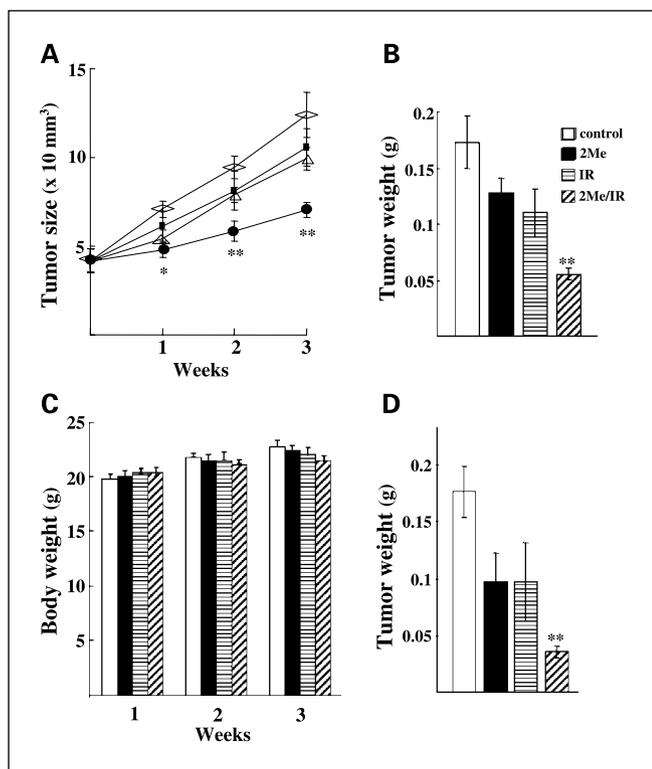


Fig. 2. Combination therapy strongly suppressed tumor progression of DLD-1 xenografts. *A*, tumor size. DLD-1 xenografts were untreated (□), treated twice weekly with 2ME (■), IR (Δ), or both (●), and evaluated by measurement of tumor volume weekly. *, *P* < 0.05 by ANOVA statistical analysis comparing four groups. *B*, tumor weight. After 3 weeks, mice were killed and tumor weights were measured. *C*, body weight. Body weights of all mice were measured weekly. Each group has six mice. Columns and points, mean; bars, SD (*A-C*). *D*, tumor weight. DLD-1 xenografts were also treated thrice weekly as in (*A*), and tumor weights were measured at day 21. Each group has four mice. Columns, mean; bars, SD. *, *P* < 0.05; **, *P* < 0.01 comparing other groups.

Western blots. Cell lysates were fractionated by 10% to 15% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and subjected to Western blots by a standard enhanced chemiluminescence method (Amersham Biosciences AB, Uppsala, Sweden).

Clonogenic assay. Cells (3×10^3) were seeded on to 60-mm plates, cultured for 1 day, pretreated with or without 2 μmol/L 2ME for 12 hours, irradiated at the indicated doses (Gy), and washed 36 hours after irradiation. After incubation for 9 days, colonies (>25 cells) were fixed in methanol, stained with Giemza, and counted. The data represent the mean ± SD of three independent experiments. Survival fraction was defined as number of colonies divided by the number of plated cells.

Confocal microscopy. DLD-1 cells were cultured on bovine serum albumin-coated coverslips overnight and treated as indicated. After fixation with cold methanol for 20 minutes, sections were soaked into 5% bovine serum albumin and incubated with anti-phosphorylated H2AX or rabbit IgG antibodies for 30 minutes. Sections were thereafter incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) for 30 minutes. Confocal imaging was done using a Zeiss Pascal (Jena, Germany) laser scanning microscope.

Statistical analysis. Statistical differences between two groups were calculated using Student's *t* test. One-way ANOVA was used to determine statistical differences between multiple groups by doing SPSS program version 10.1 (San Rafael, CA). *P* < 0.05 was considered statistically significant.

Results

Radiosensitizing effect of 2ME. Although 2ME alone induces apoptosis in a wide range of cancer cells, there is little information about its effect on radiation-induced apoptosis. Thus far, a single article reports that oral administration of 2ME potentiates the antitumor effect of IR in non-small-cell lung cancer xenografts and the authors suggest that its effect depends on wild-type p53 functions (20). As described above, 2ME induces a variety of proapoptotic signals and may enhance radiosensitivity by different mechanisms in different cell types. Based on this idea, we first investigated whether 2ME could enhance IR effects in a colon carcinoma DLD-1 cell line expressing mutant p53. In these cells, 2 μmol/L 2ME pretreatment alone induced limited cell death, whereas its combination with IR clearly enhanced cell death (Fig. 1A). The same pretreatment increased IR-induced nuclear fragmentation at similar levels, but E₂ had no effect (Fig. 1B). The number of

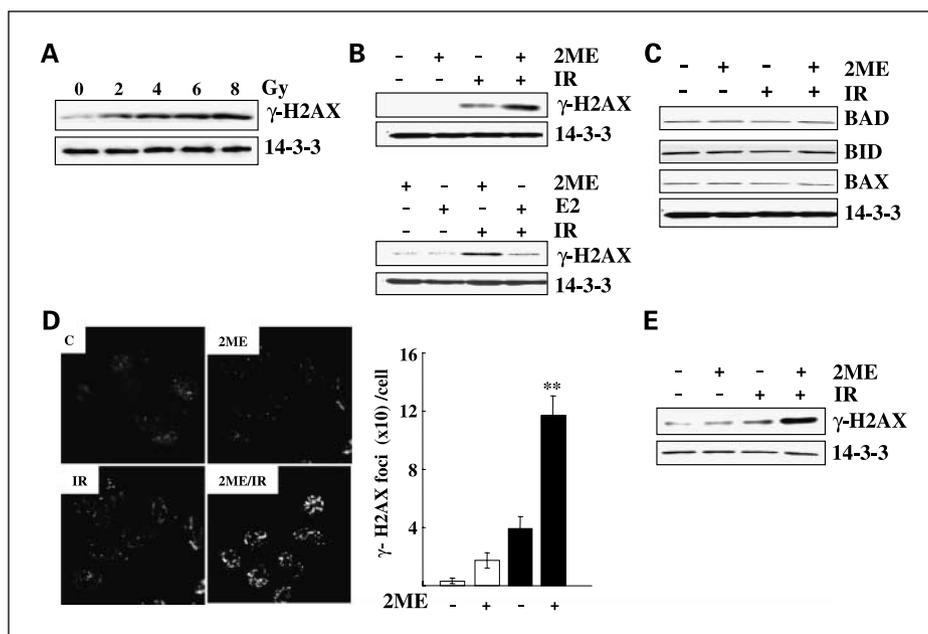
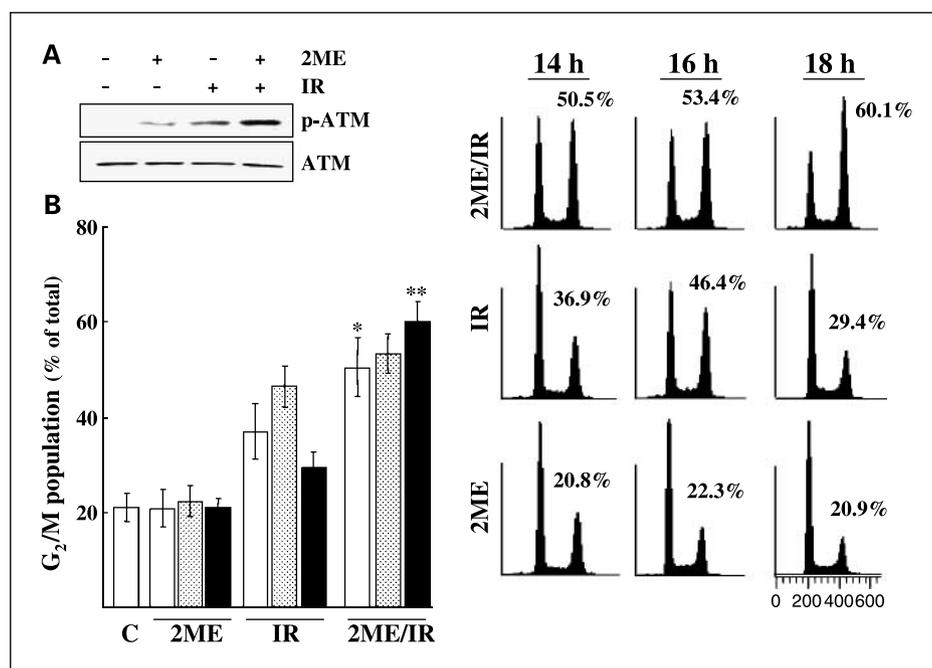


Fig. 3. Effect of 2ME on IR-induced H2AX phosphorylation. *A*, H2AX phosphorylation. Cells were harvested 30 minutes after IR at the indicated doses. *B*, H2AX phosphorylation and E₂. Cells were pretreated with 2 μmol/L 2ME or E₂ for 12 hours and harvested 30 minutes after 4 Gy irradiation. *C*, expression of proapoptotic molecules. Cells were pretreated with 2 μmol/L 2ME for 12 hours and harvested 12 hours after 4 Gy irradiation. *D*, H2AX foci. Cells were fixed 30 minutes after 4 Gy irradiation with or without 2 μmol/L 2ME pretreatment (12 hours) and H2AX foci numbers were counted. **, *P* < 0.01 comparing IR alone. *E*, H2AX phosphorylation. DLD-1 xenograft tumors were pretreated with or without 2 μmol/L 2ME for 12 hours and harvested 30 minutes after 4 Gy irradiation. 14-3-3 shows that equal amount of protein was loaded.

Fig. 4. Effect of 2ME on ATM phosphorylation and G₂-M accumulation after IR. *A*, ATM phosphorylation. Cells were treated as indicated (2 μmol/L 2ME pretreatment for 12 hours and/or 4 Gy irradiation) and harvested 30 minutes after IR. ATM expression shows equal amount of protein was loaded and no distinct change in its expression level. *B*, cell cycle analysis. Cells were treated as in (*A*), harvested at 14 (white columns), 16 (gray columns), or 18 (black columns) hours after IR, and stained with propidium iodide, and intracellular DNA content was evaluated by flow cytometry. Columns, mean; bars, SD. *, *P* < 0.05; **, *P* < 0.01 comparing IR alone. In representative data, numbers are the percentage cells at the G₂-M phase.

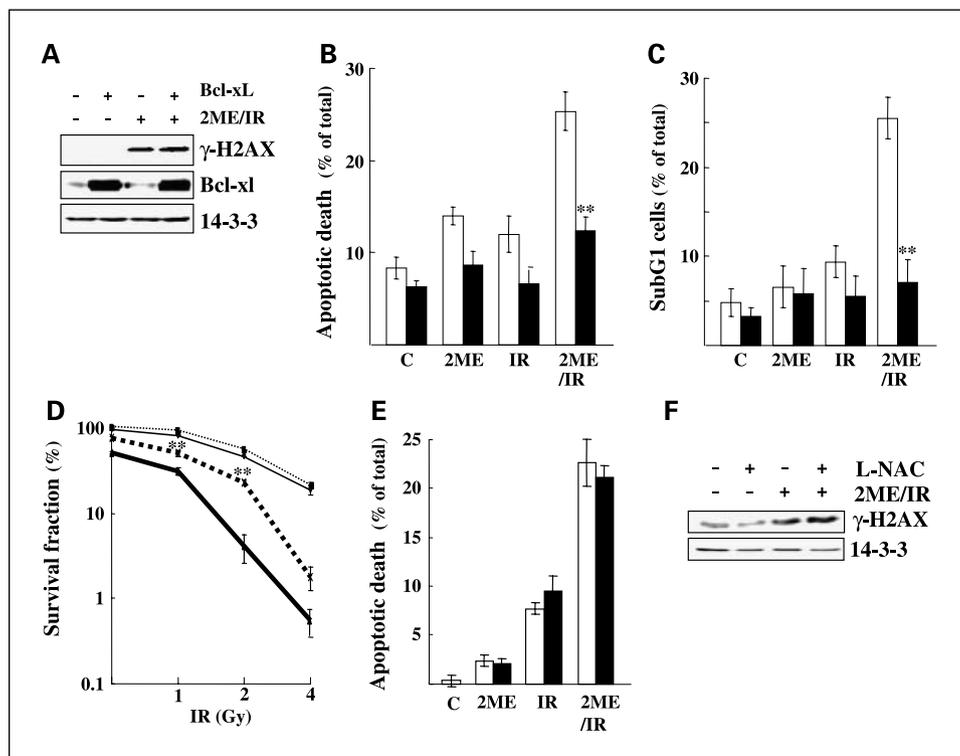


cells with nuclear fragmentation observed after combination therapy exceeded the calculated additive effect. For example, at 6 Gy irradiation, calculated survival rate is 0.89 ± 0.02 (survival rate of IR treatment) \times 0.97 ± 0.01 (survival rate of 2ME treatment) and calculated death rate is $1 - (0.89 \times 0.97) = 0.14$. However, the combination caused a much greater death rate, 0.25 ± 0.02 , indicating synergy between 2ME and IR. Clonogenic assay showed that 2ME, but not E₂, pretreatment

significantly reduced the survival fraction compared with IR alone (Fig. 1C).

We next investigated whether the radioenhancing effect of 2ME depends on pretreatment. When cells were treated with 2 μmol/L 2ME for 12 hours immediately after IR exposure, there was no enhancement (Fig. 1D), indicating that pretreatment is necessary for the radiosensitizing effect of 2ME. Importantly, 2ME pretreatment slightly increased IR-induced

Fig. 5. Effect of Bcl-xL overexpression. *A*, Bcl-xL overexpression. At 24 hours after transfection with the indicated vector, cells were pretreated with 2 μmol/L 2ME for 12 hours, harvested 48 hours after 4 Gy irradiation, and subjected to Western blot staining for the indicated proteins. *B*, DAPI staining. Mock (white columns) or Bcl-xL (black columns) transfectants were treated as indicated (2 μmol/L 2ME pretreatment for 12 hours and/or 4 Gy irradiation) and stained with DAPI 48 hours after IR. *C*, sub-G₁ population. Transfectants were treated as in (*B*), stained with propidium iodide 48 hours after IR, and subjected to flow cytometry. *D*, clonogenic assay. Mock (lines) or Bcl-xL (broken lines) transfectants were incubated with 2ME (2 μmol/L) for 12 hours, irradiated at the indicated doses, and cultured for 9 days and colony numbers (>25 cells) were counted. *E*, effect of L-NAC on combination treatment. DLD-1 cells were pretreated with 5 mmol/L L-NAC for 3 hours, thereafter treated as in (*B*), and stained with DAPI 36 hours after IR. *F*, effect of L-NAC on H2AX phosphorylation. Cells were treated with 5 mmol/L L-NAC for 3 hours, treated with 2ME/IR as in (*B*), and harvested 30 minutes after IR. 14-3-3 protein levels show the same amount of protein loaded in each lane (*A* and *F*). Columns and points, mean; bars, SD. **, *P* < 0.01 compared with mock transfectants (*B-E*).



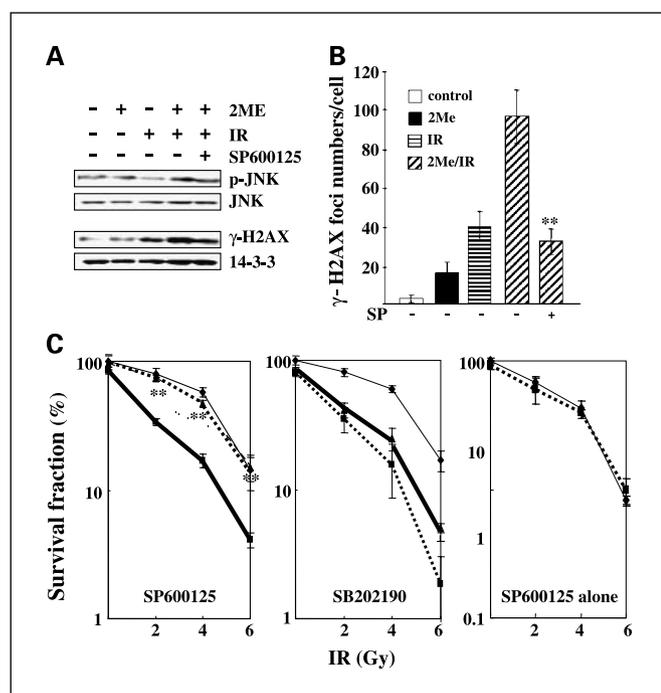


Fig. 6. Effect of SP600125. **A**, JNK and H2AX phosphorylation. Cells were treated with or without 10 μ mol/L SP600125 for 3 hours before 2 μ mol/L 2ME pretreatment and/or 4 Gy irradiation and harvested 30 minutes after IR. 14-3-3 and JNK expression shows that equal amount of protein was loaded and no distinct change in their expression level. **B**, γ -H2AX foci. Cells were treated as in (A), fixed 30 minutes after 4 Gy irradiation, and examined by confocal microscopy. **C**, clonogenic assay. Cells were treated with IR alone (*thin lines*), its combination with 2ME (*thick lines*), or 10 μ mol/L SP600125 or SB202190 before combination treatment (*broken lines*) as in (A) and fixed 9 days after IR. As a control study, cells were treated with SP600125 alone for 3 hours before IR exposure. Colony numbers (>25 cells) were counted. *Columns and points*, mean of three separate experiments; *bars*, SD. **, $P < 0.01$ compared with combination treated cells.

cell death in normal lymphocytes, but the increase was much less than in DLD-1 cells (Fig. 1D).

Anticancer effect of combination therapy against DLD-1 xenografts. We next determined whether the combination therapy is actually effective in nude mice. Based on our pilot studies, once DLD-1 tumor xenografts reached a minimal size of $45 \pm 10 \text{ mm}^3$ (approximately 10 days after cell inoculation), they were treated with 2ME (3 \times 80 nmol) and/or IR exposure (4 Gy each fraction) twice weekly. Treatment with 2ME or IR exposure alone caused partial tumor growth delay compared with control tumors, whereas combined therapy significantly retarded tumor growth (Fig. 2A). On day 21, the mean tumor weights of the combined therapy group were significantly reduced compared with the other groups ($P < 0.0001$; Fig. 2B). Importantly, no distinct difference in body weight was observed among the four groups (Fig. 2C). In addition, there were no apparent phenotypic abnormalities in the mice related to treatment and pathologic examination of liver, kidney, and stomach from control or combined therapy groups did not show any distinct abnormality (data not shown). We also obtained similar results of combination therapy (thrice weekly; Fig. 2D). These data strongly suggest that 2ME consistently increased the radiosensitivity of DLD-1 cells *in vivo*.

2ME enhanced γ -H2AX after IR exposure. We next studied the molecular mechanism(s) by which 2ME enhances radiosensitivity in DLD-1 cells. IR exposure immediately induces DNA double-strand breaks, which results in cell cycle arrest and/or apoptosis. We hypothesized that 2ME might affect IR-induced DNA damage and enhance radiation-induced cell death. To explore this possibility, we determined whether 2ME affected IR-induced phosphorylation of H2AX (γ -H2AX), which is a landmark of double-strand breaks (21, 22). In DLD-1 cells, H2AX was clearly phosphorylated 0.5 hours after

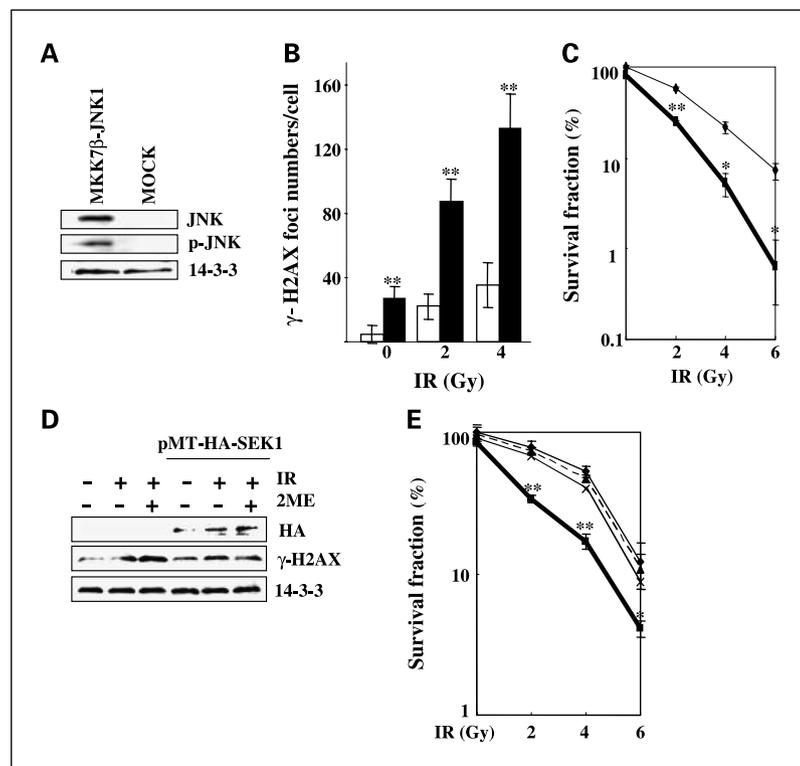


Fig. 7. Effect of JNK transfection. **A**, constitutively active JNK overexpression. Cells were transfected with mock or pEF-Flag-MKK7 β -JNK1 expression vector and harvested 24 hours after transfection. **B**, γ -H2AX foci. Mock (*white columns*) and MKK7 β -JNK1 (*black columns*) transfectant cells were fixed 30 minutes after 2 or 4 Gy irradiation, and their γ -H2AX foci were examined by confocal microscopy. **C**, clonogenic assay. Cells were transfected with mock (*thin line*) or pEF-Flag-MKK7 β -JNK1 (*thick line*), exposed to IR, and fixed 9 days after irradiation. **D**, dominant-negative mutant SEK1 overexpression. Cells were transfected with mock or pMT-HA-SEK1 expression vector, treated with 2ME (2 μ mol/L) for 12 hours, and harvested 30 minutes after 4 Gy irradiation. HA is SEK1 (MKK4) overexpression, which inhibits 2ME-mediated γ -H2AX enhancement. **E**, clonogenic assay. Cells were transfected with mock (*lines*) or pMT-HA-SEK1 (*broken lines*), exposed to IR with (*thick line*) or without (*thin line*) 2ME pretreatment, and fixed 9 days after irradiation. Colony numbers (>25 cells) were counted. *Columns and points*, mean of three separate experiments; *bars*, SD. 14-3-3 shows that equal amount of protein was loaded (A and D). *, $P < 0.05$; **, $P < 0.01$ compared with mock transfectants.

2 to 8 Gy irradiation (Fig. 3A), and 2ME pretreatment further increased the phosphorylation level (Fig. 3B). In contrast, E₂ failed to enhance IR-induced phosphorylation at H2AX. Importantly, the 2ME treatment did not affect expression of proapoptotic molecules, such as BAD, BID, and BAX, thereby excluding a modification of their expression levels (Fig. 3C). To confirm the effect of 2ME on IR-induced double-strand breaks, we evaluated γ -H2AX foci after IR exposure and found that they were strongly increased by 2ME pretreatment (Fig. 3D),

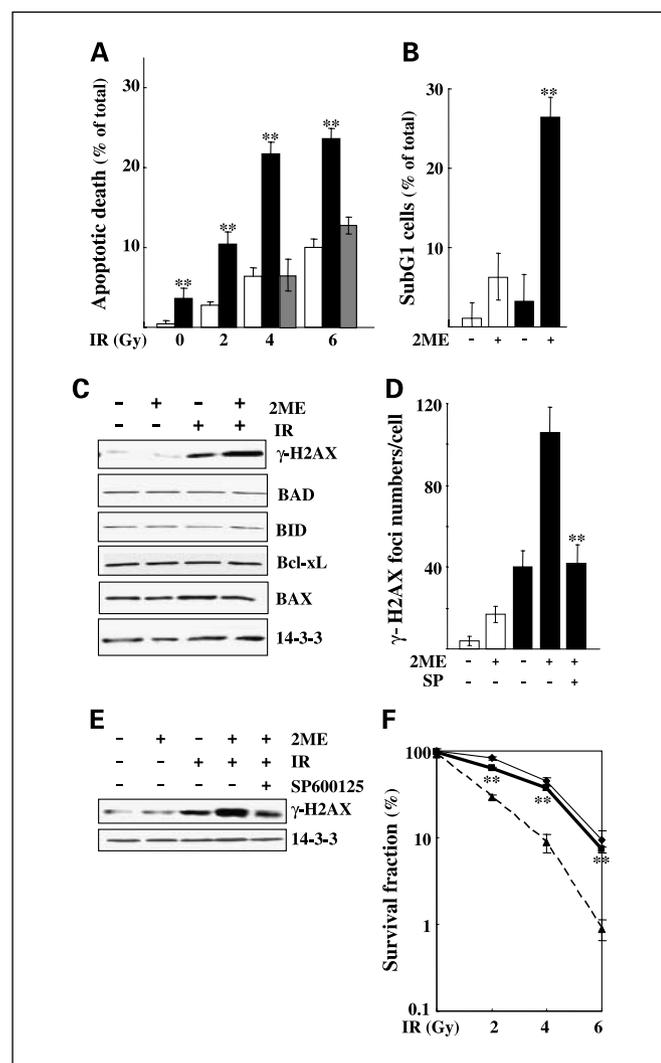


Fig. 8. Combination therapy in colon cancer HCT-15 cells. *A*, DAPI staining. Cells were pretreated with 0.5 μ mol/L 2ME (black columns) or E₂ (gray columns) for 12 hours, irradiated at the indicated doses, cultured without 2ME or E₂ for 48 hours, and stained with DAPI. *B*, Sub-G₁ population. Cells were treated as in (*A*), stained with propidium iodide 48 hours after IR (black columns), and subjected to flow cytometry. **, $P < 0.01$ comparing IR alone (*A* and *B*). *C*, expression of proapoptotic molecules. Cells were pretreated with 0.5 μ mol/L 2ME for 12 hours and harvested 12 hours after 4Gy irradiation. *D*, γ -H2AX foci. Cells were treated with or without 10 μ mol/L SP600125, thereafter treated as in (*A*), and fixed 30 minutes after IR (black columns), and γ -H2AX foci were counted. *E*, H2AX phosphorylation. Cells were treated with or without 10 μ mol/L SP600125 for 3 hours before 0.5 μ mol/L 2ME pretreatment and/or 4 Gy irradiation and harvested 30 minutes after IR. *F*, clonogenic assay. Cells were preincubated without 2ME (thin line), with 2ME (broken line), or SP600125 before 2ME pretreatment (thick line) as in (*D*) and fixed 9 days after IR at the indicated doses. Colony numbers (>25 cells) were counted. Columns and points, mean of three separate experiments; bars, SD (*A*, *B*, *D*, and *F*). 14-3-3 shows that equal amount of protein was loaded (*C* and *E*). **, $P < 0.01$ compared with combination treated cells (*D* and *F*).

indicating that 2ME specifically enhanced IR-induced DNA damage and implying a role of DNA damage for its enhancing effect on radiosensitivity. In addition, the 2ME-mediated enhancing effect on γ -H2AX was also observed in DLD-1 xenografts after IR exposure (Fig. 3E), suggesting that the 2ME-mediated inhibitory effect on tumor progression may involve a similar mechanism to that observed *in vitro*.

IR-induced DNA damage initially activates ATM kinase (23, 24). If 2ME enhances IR-induced DNA damage, it should also increase ATM phosphorylation. Indeed, 2ME pretreatment clearly augmented increased ATM phosphorylation after IR (Fig. 4A). Furthermore, 2ME pretreatment clearly augmented arrest at the G₂-M phase and delayed cell cycle progression after IR because cells treated with IR alone already had begun to proceed with cell cycle progression from arrest at the G₂-M phase 18 hours after IR, whereas the majority of the population (>60%) of cells treated with 2ME/IR still remained at the G₂-M phase (Fig. 4B). These data support our idea that 2ME enhanced IR-induced DNA damage.

Overexpression of Bcl-xL canceled the enhancing effects of 2ME on IR. We next investigated whether the 2ME-mediated enhancing effect on cell death involves enhanced apoptosis. Gene transfection of the pcDNA3-Bcl-xL expression vector clearly increased Bcl-xL expression (Fig. 5A). At 48 hours after 4 Gy irradiation, cell death (data not shown), cells with nuclear fragmentation (Fig. 5B), and sub-G₁ cells (Fig. 5C) were clearly decreased by Bcl-xL overexpression. In addition, Bcl-xL overexpression suppressed 2ME-mediated enhanced radiosensitivity (Fig. 5D), suggesting that the 2ME-mediated radiosensitizing effect was from enhanced apoptosis. However, it did not affect 2ME-mediated enhanced γ -H2AX (Fig. 5A), which may exclude the possibility that the 2ME-mediated increase of γ -H2AX was caused by enhanced apoptosis.

L-N-acetylcystein barely affected 2ME-mediated enhancing effect on IR. To further explore the molecular mechanism(s) by which 2ME radiosensitizes DLD-1 cells, we investigated whether oxidative stress is crucial for combination therapy-induced cell death because 2ME induces ROS generation by inhibiting superoxide dismutase (8), and ROS may induce oxidative DNA damage (25). To explore this, the free radical scavenger L-N-acetylcystein (L-NAC) was incubated with the cells before combination treatment. L-NAC inhibited the 2ME-mediated enhancing effect on neither apoptotic cell death nor γ -H2AX (Fig. 5E and F), implying that oxidative stress was marginal for the augmented cell death and DNA damage.

JNK inhibitor canceled the effects of 2ME. We next examined the effects of a specific JNK inhibitor SP600125. As described above, several reports indicate that 2ME activates JNK, which is persistently activated in irradiated cells and considered as a pivotal mediator of apoptotic signals (26, 27). 2ME pretreatment only slightly increased JNK phosphorylation, but it was further enhanced by IR exposure, and the increase was clearly inhibited by SP600125 (Fig. 6A). Importantly, SP600125 pretreatment almost completely canceled the enhancing effect of 2ME on both γ -H2AX (Fig. 6A and B) and radiosensitivity (Fig. 6C). However, a p38 inhibitor SB202190 did not show inhibitory effect on the 2ME-mediated enhancing radiosensitivity and SP600125 alone barely affected radiosensitivity (Fig. 6C). Furthermore, DLD-1 cells transfected with the pEF-Flag-MKK7 β -JNK1 vector expressed phosphorylated JNK abundantly (Fig. 7A) and strongly enhanced γ -H2AX after IR

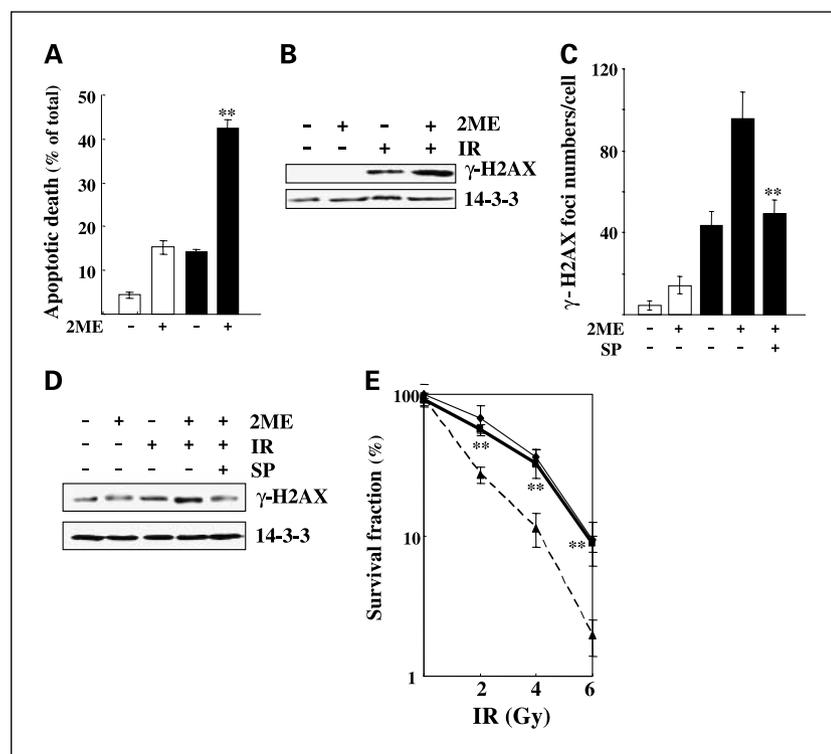


Fig. 9. Combination therapy in colon cancer HCT-8 cells. **A**, DAPI staining. Cells were pretreated with 2 $\mu\text{mol/L}$ 2ME for 12 hours, exposed to 4 Gy irradiation (*black columns*), cultured without 2ME for 48 hours, and stained with DAPI. **, $P < 0.01$ comparing IR alone. **B**, H2AX phosphorylation. Cells were treated with 2 $\mu\text{mol/L}$ 2ME pretreatment and/or 4 Gy irradiation and harvested 30 minutes after IR. **C**, γ -H2AX foci. Cells were treated with or without 10 $\mu\text{mol/L}$ SP600125 for 3 hours, thereafter treated as in (**B**), and fixed 30 minutes after 0 (*white columns*) or 4 Gy irradiation (*black columns*), and foci were counted. **D**, H2AX phosphorylation. Cells were treated with or without 10 $\mu\text{mol/L}$ SP600125 for 3 hours before 2 $\mu\text{mol/L}$ 2ME pretreatment and/or 4 Gy irradiation and harvested 30 minutes after IR. 14-3-3 shows that equal amount of protein was loaded (**B** and **D**). **E**, clonogenic assay. Cells were preincubated without 2ME (*thin line*), with 2ME (*broken line*), or SP600125 before 2ME pretreatment (*thick line*) as in (**D**) and fixed 9 days after IR. Colony numbers (>25 cells) were counted. *Columns* and *points*, mean of three separate experiments; *bars*, SD (**A**, **C**, and **E**). **, $P < 0.01$ comparing combination treated cells (**C** and **E**).

exposure (Fig. 7B) and showed a greater radiosensitivity than mock transfectants (Fig. 7C). We also investigated the roles of JNK in DLD-1 cells transfected with pMT-HA-SEK1 (MKK4) vector (28), which dominant negatively affects JNK activity. Importantly, 2ME-mediated γ -H2AX enhancement (Fig. 7D) and radiosensitization (Fig. 7E) were mostly inhibited in the pMT-HA-SEK1 transfectants. These results strongly suggest the pivotal role of JNK in the 2ME-mediated radiosensitivity enhancement.

Anticancer effect of combination therapy in HCT-15 and HCT-8 cells. To address whether combination therapy is effective in other colon cancer cells, we tested its effect in two other colon cancer cell lines. Combination therapy strikingly augmented apoptotic cell death and sub- G_1 in HCT-15 cells (Fig. 8A and B). As observed in DLD-1 cells, 2ME did not affect expression levels of proapoptotic molecules, but it strongly enhanced H2AX phosphorylation (Fig. 8C). In addition, SP600125 substantially decreased the enhancing effect of 2ME on both IR-induced γ -H2AX (Fig. 8D and E) and radiosensitivity (Fig. 8F). Similarly, 2ME enhanced IR effects in another colon cancer cell line, HCT-8 (i.e., augmented apoptotic cell death, γ -H2AX, and radiosensitivity; Fig. 9A-E). These events were clearly inhibited by SP600125 (Fig. 9C-E). Collectively, our findings suggest that 2ME may represent a JNK-mediated radiosensitizer probably in a broad array of colon cancer cells.

Discussion

We have shown that 2ME strongly enhances radiosensitivity in three different colon cancer cell lines. Although radiation therapy is not commonly chosen to treat primary lesions, enhancement of radiosensitivity could make it the first choice.

We also showed that the combination of 2ME and IR strongly inhibits tumor progression of DLD-1 xenografts. The 2ME therapeutic potential is strengthened because it is a physiologic metabolite of estrogen and is effective in concentrations much less than those used independently, allowing us to consider that this combination therapy may be clinically applicable. Although 2ME induces apoptosis in a variety of carcinomas, there is little information about its radiosensitizing effect. Another investigator reported that 2ME somehow enhances the antitumor effect of IR on xenografts and suggested that this effect requires wild-type p53 functions (20). However, in our studies, the enhancing effect of 2ME on IR was totally independent of p53 functions because radiosensitivity was enhanced similarly in three colon cancers, mutant p53-expressing DLD-1 and HCT-15, and wild-type p53-expressing HCT-8 cells. Our data rather suggest that the effects may result from increased DNA damage, which was evaluated by γ -H2AX, its foci, ATM phosphorylation, and re-entry into the cell cycle. Although γ -H2AX was also increased by induction of apoptosis, the 2ME-mediated increase is likely to be caused by enhanced double-strand breaks because γ -H2AX was not reversed by Bcl-xL overexpression, which substantially reversed cell death. Therefore, 2ME more likely enhances radiosensitivity mostly through increased double-strand breaks after IR.

We show that the 2ME-mediated radiosensitizing effect depends on pretreatment because there was no enhancement when DLD-1 cells were treated with 2ME immediately after IR (Fig. 1D). Although 2ME elevates ROS generation by inhibiting superoxide dismutase in several cancer cells (7-9), we could not detect any significant increase of ROS generation in the colon cancers we examined (data not shown), and an anti-scavenger agent L-NAC showed no inhibitory effect. Therefore,

ROS-mediated DNA damage seems to be marginal for the radioenhancing effect. In contrast, SP600125 or overexpression of a dominant-negative mutant SEK1 strongly blocked the effects of 2ME on IR. Indeed, 2ME pretreatment alone only slightly increased JNK phosphorylation, but after IR, it further increased JNK phosphorylation and γ -H2AX (Fig. 6A). This strongly suggests that augmented JNK activation may induce more extensive DNA damage and subsequently induce cell death. This suggestion is basically in agreement with previous studies showing pivotal roles for JNK in IR-induced apoptosis (26, 27, 29, 30) and the possible mechanisms by which JNK induces DNA damage (31, 32). Although we identify JNK activation among a variety of intracellular signals of 2ME, it still remains uncertain how JNK enhances IR-induced DNA damage and precisely which molecular mechanisms are involved. To

answer these questions, we are currently studying the roles of JNK activation in the enhancement of DNA damage.

In conclusion, our study identifies 2ME as a strong radiosensitizing agent in colon cancer cells and suggests the mechanism is through JNK-mediated enhanced DNA damage. Its dependency on JNK activation makes 2ME unique among radiosensitizers. Considering that 2ME is a physiologic metabolite of estrogen, our findings facilitate the development of improved anticancer strategies, especially against colon cancer cells.

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