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A Novel Anticancer Ribonucleoside, 1-(3-C-Ethynyl-β-D-ribo-pentofuranosyl)Cytosine, Enhances Radiation-Induced Cell Death in Tumor Cells

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1-(3-C-Ethynyl-β-D-ribo-pentofuranosyl)cytosine (ECyd, TAS106) is a newly developed anti-tumor agent that targets RNA synthesis. We report here that a low dose of ECyd induces radiosensitization of caspase-dependent apoptosis and reproductive cell death in cells of the gastric tumor cell lines MKN45 and MKN28 and murine rectum adenocarcinoma Colon26. Flow cytometry demonstrated that TAS106 induced the abrogation of the X-ray-induced G_2/M checkpoint. Western blot analysis showed that X rays increased the expression of cyclin B1, phospho-Cdc2 and Wee1, whereas co-treatment with X rays and TAS106 decreased the expression of these cell cycle proteins associated with the G_2/M checkpoint. Furthermore, TAS106 was shown to decrease the radiation-induced expression of survivin but not Bcl2 and $BclX_L$ regardless of TP53 status and cell type. Overexpression of wild-type survivin in MKN45 cells inhibited the induction of apoptosis induced by co-treatment with X rays and TAS106. These results suggest that TAS106 enhances X-ray-induced cell death through down-regulation of survivin and abrogation of the cell cycle machinery. © 2004 by Radiation Research Society

INTRODUCTION

Ionizing radiation is known to cause cell cycle arrest in the G_2/M phase as well as cell death. DNA double-strand breaks induced by radiation are recognized by ataxia telangiectasia mutated (ATM), which inhibits the passage of DNA-damaged cells from G_2 into the M phase. ATM is

required for the activation of Chk1/2 in response to DNA damage, followed by the phosphorylation of Cdc25C phosphatase on Ser216. The phosphorylation of Cdc25C phosphatase creates a binding site for 14-3-3 proteins. The 14-3-3-bound phosphatase is then sequestered outside of the nucleus and cannot dephosphorylate and activate the mitosis-promoting Cdc2-cyclin B1 complex (*1*).

Abrogation of the G_2/M checkpoint often leads to a marked increase in the sensitivity of cells to ionizing radiation and some types of chemotherapy. 7-Hydroxystaurosporine (UCN-01) has been reported to enhance the antitumor activities of mitomycin C (*2*) and ionizing radiation (*3–6*) by abrogating the G_2/M checkpoint functions through the inhibition of a kinase presenting upstream of Cdc25C, Cdc2 and Wee1. An indolocarbazole inhibitor (SB-218078) against Chk1 was also reported to abrogate the G_2/M arrest caused by γ-ray-induced or topotecan-induced DNA damage and to enhance apoptosis (*7*). Caffeine is also known to be a radiosensitizing agent (*8–10*) that attenuates the radiation-induced G_2/M checkpoint by inhibiting radiation-induced activation of ATM and ataxia telangiectasia mutated and Rad3-related (ATR) (*10*). Furthermore, abrogation of DNA damage-induced S and G_2/M checkpoints by antisense inhibition of Chk2 has been shown to enhance radiation-induced apoptosis in HEK-293 cells (*11*). These results suggest a strong relationship between radiosensitization and inhibition of the G_2/M checkpoint in various tumor cell lines, irrespective of TP53 status. However, less is known about how abrogation of the G_2/M checkpoint leads to apoptosis.

Survivin, which belongs to the inhibitors of apoptosis protein (IAP) family with a domain designated the baculovirus IAP repeat (BIR), has been found to be intensively expressed in the G_2/M phase (*12*). Survivin is located in kinetochores of metaphase chromosomes and in the central spindle midzone in anaphase (*13*). Several reports have consistently shown that the overexpression of survivin inhibits cell death induced by various stimuli of apoptosis.

Targeting experiments using an antisense oligodeoxyribo-nucleotide for survivin, a Thr34→Ala or Cys84→Ala dominant negative mutant induced the loss of the functions of survivin, followed by the enhancement of apoptosis induced by spontaneous or genotoxic stimuli or the inhibition of cell proliferation (*14–20*). This ability of survivin to inhibit apoptosis has been considered to be due to attenuation of the activities of caspases 3 and 7 by binding with the BIR domain of survivin (*21, 22*). Recently, survivin was reported to inhibit not only caspase-dependent apoptosis but also caspase-independent cell death as a result of mitotic catastrophe (*23*). Furthermore, it has been demonstrated that the cell cycle periodicity of survivin expression is controlled by a ubiquitin-dependent destruction system, and >40-fold up-regulated survivin is observed in the G_2/M phase (*24*). Survivin undergoes cell cycle-dependent phosphorylation on Thr34 by a Cdc2-cyclin B1 complex, and the loss of this phosphorylation results in the dissociation of the survivin-caspase 3 complex on the mitotic apparatus and caspase 3-dependent apoptosis of cells traversing mitosis (*17–19*). On the other hand, phosphorylation of Bcl2, another anti-apoptosis protein, in G_2/M-phase-arrested cells after photodynamic therapy with hypericin involves a Cdc2-mediated signal and delays the onset of apoptosis (*25*). These experiments led us to assume that survivin or Bcl2 played a key role in the induction of apoptosis by abrogation of the DNA damage-induced G_2/M checkpoint.

1-(3-C-Ethynyl-β-D-*ribo*-pentofuranosyl) cytosine (ECyd, TAS106) has recently been developed as a novel antitumor agent (*26*). TAS106 (ECyd) is rapidly phosphorylated to ECyd 5′-triphosphate (ECTP), which is essential for its cytotoxicity (*26–31*). The strong toxicity of ECTP for tumor cells is due to its inhibition of RNA synthesis. Because of the mechanism of action of TAS106, it is possible that a low dose of TAS106 would enhance radiation-induced apoptosis through the down-regulation of protein expression related to a cell cycle checkpoint or an anti-apoptosis function. In this study, we investigated the effects of TAS106 on radiation-induced cell death and the G_2/M checkpoint in gastric tumor cells and murine rectum tumor cells using a low dose of TAS106, which did not induce cell death itself.

MATERIALS AND METHODS

Materials

1-(3-C-Ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (TAS106) was synthesized as described elsewhere (*26*). Antibodies against Cdc2, cyclin B1, Wee1, Bcl2, BclX$_L$ and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-Cdc25 and phospho-Cdc2 were from Cell Signaling Technology Inc. (Beverly, MA), and the antibody against survivin was from Alpha Diagnostic International, Inc. (San Antonio, TX). Other reagents were purchased from Wako Chemical Co. (Osaka, Japan).

Cell Culture and Treatment of TAS106 and X Irradiation

Human gastric adenocarcinoma MKN45 (TP53 wild-type) and MKN28 (TP53 mutation) cells and murine rectum adenocarcinoma Colon26 cells were grown in monolayer cultures at 37°C in RPMI 1640 medium (Gibco-BRL/Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum. The MKN45, MKN28 and Colon26 cells, which were pretreated with 0.1, 1 and 5 μ*M* of TAS106, respectively, were irradiated with an X-ray generator (2.0-mm aluminum filter, 200 kVp, 20 mA, Shimadzu HF-320, Kyoto, Japan) at a dose of 3.03 Gy/min, which was determined using a Fricke chemical dosimeter. Cells (1×10^6 cells/ml) were incubated in growth medium at 37°C for 1 h before X irradiation. For treatment with benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) (Peptide Institute, Tokyo, Japan), cells were incubated in growth medium with the inhibitor at 50 μ*M* for 1 h before X irradiation.

Fluorescence Microscopic Observation of Apoptotic Cells

Cells (5×10^5) incubated for the indicated periods after X irradiation without or with drugs were collected by centrifugation at 1,000 rpm for 5 min at 4°C. The pellet was washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline [PBS(–)] and fixed with 1% glutaraldehyde/PBS(–) solution. For fluorescence microscopy, the fixed cells were washed and resuspended in PBS(–). An aliquot was stained with propidium iodide (Sigma Chemical, St. Louis, MO) in PBS(–) at 40 μg/ml for 15 min in the dark. The stained cells were placed on a microscope slide and gently covered with a cover slip. Fluorescence microscopic observation was performed using an Olympus BX50 microscope (Olympus, Tokyo, Japan) with reflected-light fluorescence (*32, 33*).

Clonogenic Assay

The MKN45, MKN28 and Colon26 cells, which were treated with 0.1, 0.5 and 0.5 μ*M* of TAS106 at 37°C for 1 h, respectively, were irradiated with X rays at doses from 0 to 9 Gy, and then 100 to 30,000 cells were seeded on 6-cm petri dishes containing the medium with TAS106, followed by incubation at 37°C for 36 h. After the medium was changed to fresh medium without TAS106, the cells were subsequently cultured. After 7 days, the cells were fixed with methanol and stained with Giemsa stain. Only colonies containing more than 50 cells were scored as surviving cells. After the surviving fraction at each dose was calculated with respect to the plating efficiency of the nonirradiated control, the dose–response curves were plotted.

Cloning of Survivin cDNA, Plasmid Construction and Expression

Human survivin cDNA was obtained by reverse transcription-PCR of RNA derived from HeLa cells with the following primers based on GenBank accession number U75285 (forward, 5′-GCATGGGTGCCCC GACGTTG-3′, reverse, 5′-GCTCCGGCCAGAGGCCTCAA-3′). PCR products were cloned into pCR 2.1 TOPO vector using the TOPO TA cloning system according to the manufacturer's instructions (Gibco-BRL/Invitrogen). Wild-type survivin cDNA was cloned into the *Eco*RI site of pCIneo expression vector (Clontech BD Biosciences, Palo Alto, CA). In transfection of the plasmid construction, LipofectAMINE Plus (Gibco-BRL/Invitrogen) was used to transfect 0.4 μg of pCIneo-survivin vector and 0.1 μg of pQBI-GFP vector (Nippon Gene, Tokyo, Japan) per 35-mm dish of MKN45 cells (1.5×10^5/dish) according to the manufacturer's instructions. The cells transfected with the expression vector were incubated for 36 h after the treatment with TAS106 and/or X rays and then apoptosis and cell cycle status were examined by fluorescence microscopy and flow cytometry, respectively.

Agarose Gel Electrophoresis for Apoptosis

Cells (2.0×10^7) exposed to X rays and/or TAS106 for 36 h were collected by centrifugation at 1,000 rpm for 5 min at 4°C. The pellet was washed with PBS(–). DNA fragmentation assay was performed according to the method of Ramakrishnan and Catravas (*34*). Briefly, lysis buffer (0.2% Triton X-100, 10 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA) was added to the cell pellet followed by centrifugation at 15,000 rpm for 30 min at 4°C. DNA was extracted from the supernatant by phenol:chloroform:isoa-
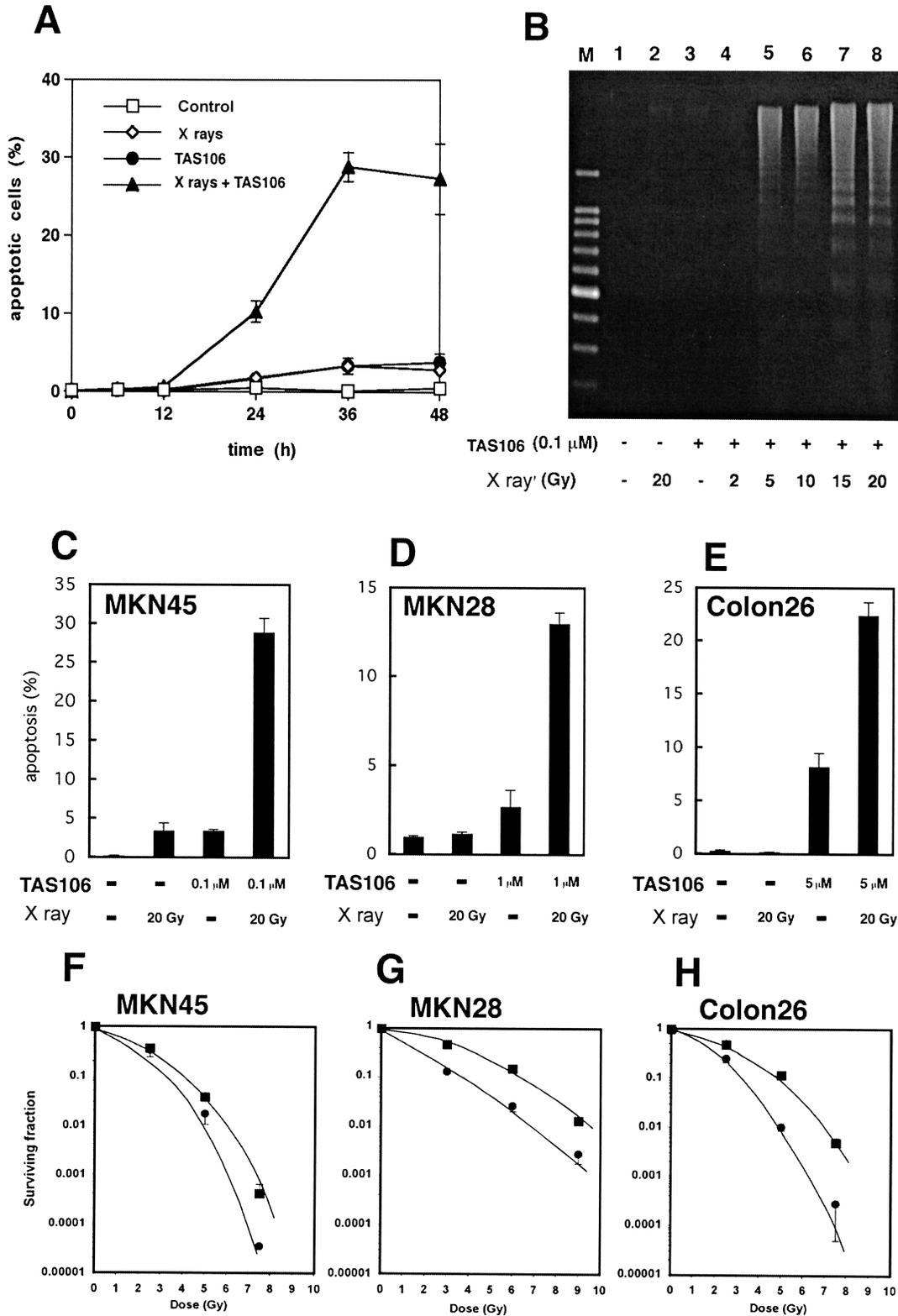
FIG. 1. TAS106 enhances X-ray-induced apoptosis and reproductive death in MKN45, MKN28 and Colon26 cells. Panel A: KN45 cells treated with 0.1 μ*M* TAS106, 20 Gy X rays, or X rays (20 Gy) + TAS106 (0.1 μ*M*) were incubated for several hours. Cells were collected at the indicated times and stained with propidium iodide after fixation. At least 200 cells were scored for each determination for apoptotic cells. Panel B: DNA fragmentation was measured 48 h after treatment with various doses of X rays in the presence of TAS106. Panels C and D: Radiosensitization of apoptosis and abrogation of the G_2/M checkpoint by TAS106 irrespective of TP53 status and cell line. After treatment with TAS106, X rays or X rays + TAS106, cells were collected at 48 h for MKN45 cells (panel C), 24 h for MKN28 cells (panel D), and 36 h for Colon26 (panel E) and stained with propidium iodide after fixation. At least 200 cells were scored by fluorescence microscopy for each determination for apoptotic cells. Columns and bars represent means ± SEM. Clonogenic assay for X-irradiated MKN45 (panel F), MKN28 (panel G) and Colon26 cells (panel H). (■) X rays only, (●) X rays + TAS106. Data are expressed as means ± SEM for three experiments.

myl alcohol = 25:24:1 and analyzed by 2% agarose gel electrophoresis. DNA fragments in the gel were detected by staining with ethidium bromide (Sigma).

Analysis of Cell Cycle by Flow Cytometry

Cells exposed to TAS106 and/or X rays were harvested by treatment with trypsin-EDTA solution. After washing with ice-cold PBS(–), the cells were fixed with ice-cold 70% ethanol and stored at 4°C for 2 h and RNA was hydrolyzed with 2 µg/ml RNase A (Boehringer Mannheim GmbH, Germany) at 37°C for 30 min. Then the cells were stained with propidium iodide (Sigma) for 20 min. The DNA content of cells was analyzed using an EPICS ELITE flow cytometer (Coulter, Miami, FL).

SDS PAGE and Western Blotting

SDS PAGE and Western blotting were performed as described elsewhere (*33*). At the indicated times after X irradiation, cells (2×10^6) were collected by centrifugation at 1,000 rpm for 5 min at 4°C. The pellet was washed with PBS(–) and resuspended in 100 µl of Laemmli's sample buffer (62.5 m*M* Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue) and sonicated twice for 30 s at ice-cold temperature. Proteins in the solution were separated by SDS-PAGE after boiling for 3 min and transferred onto a nitrocellulose membrane (Advantec Toyo, Tokyo, Japan). The membrane was probed with Cdc25, phospho-Cdc25, Cdc2, phospho-Cdc2, cyclin B, Wee1, Bcl2, BclX$_L$, actin or survivin in TBST buffer (10 m*M* Tris-HCl, pH 7.4, 0.1 *M* NaCl, 0.1% Tween-20) containing 5% nonfat milk or 5% BSA. These were detected by a method using HRP-conjugated anti-rabbit, anti-mouse or anti-goat IgG antibodies with a chemiluminescence detection kit (Boehringer Mannheim GmbH).

Cdc2-Associated H1 Kinase Assay

Measurements of cyclin-dependent kinase-associated H1 kinase for Cdc2 were performed by a slight modification of the method described by Fortugno *et al.* (*13*). Cells were lysed in lysis buffer (50 m*M* Hepes/NaOH, pH 7.4, 150 m*M* NaCl, 1% Triton X-100, 1 m*M* dithiothreitol, 50 m*M* sodium fluoride, 50 m*M* β-glycerophosphate, 1 m*M* sodium *o*-vanadate, 1 m*M* EDTA, 1 m*M* phenyl methylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) for 20 min at 4°C. The cell lysate was centrifuged at 15,000*g* for 10 min at 4°C. The protein content in the supernatant was determined using a Bio-Rad protein assay kit (Hercules, CA). Then 400 µg of protein was added to protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Arlington Heights, IL), preassociated with the anti-Cdc2 antibody, and mixed gently for 2 h at 4°C. The immunoprecipitates were washed twice with lysis buffer and twice with wash buffer (50 m*M* Hepes/NaOH, pH 7.4, 10 m*M* MgCl$_2$, 1 m*M* dithiothreitol) and incubated with 40 µl of wash buffer containing 10 µg of histone H1 (Boehringer Mannheim GmbH), 10 µ*M* ATP, 92.5 TBq [γ-^{32}P]ATP (185 TBq/mmol, Amersham Pharmacia Biotech) for 10 min at 30°C. Each sample was mixed with 20 µl of 3× SDS-sample buffer to stop the reaction, heated for 3 min at 95°C and subjected to SDS-PAGE. The gel was dried and analyzed with a BAS1000 Image Analyzer (Fuji Photo Film, Tokyo, Japan).

RESULTS

### TAS106 Enhances Radiation-Induced Cell Death in Tumor Cells

Figure 1A shows the effects of X rays, TAS106 and X rays + TAS106 on apoptosis in MKN45 cells. Either X irradiation or TAS106 alone induced a small number of apoptotic cells at 24 h and a plateau phase of low-level apoptosis of less than 5% between 36 h and 48 h. In con-

trast, the number of apoptotic cells increased significantly in MKN45 cells treated with X rays plus TAS106 for 24 h and produced about 30% apoptotic cells at 36 h after treatment. As a biochemical marker of apoptosis, ladder-like DNA fragmentation was also induced by the treatment of cells with X-ray doses of more than 5 Gy plus TAS106 but was not induced when cells were treated with either X rays or TAS106 alone as shown in Fig. 1B. To examine whether this radiosensitization of apoptosis by TAS106 depended on TP53 status or cell type, in addition to MKN45 cells (TP53 wild-type), we tested the effect of a low dose of TAS106 on apoptosis in MKN28 cells (TP53 mutation) and Colon26 cells (murine rectum adenocarcinoma). In Fig. 1D and E, similar enhancement of radiation-induced apoptosis by TAS106 can be observed in MKN28 cells and Colon26 cells, although the treatment with TAS106 alone at least for 48 h induced little apoptosis (less than 10%) in MKN28 and Colon26 cells (data not shown). Furthermore, to evaluate whether TAS106 enhanced radiation-induced reproductive cell death, colony-forming ability was examined. As shown in Fig. 1F, G and H, significant radiosensitization was observed in all cell lines after treatment with TAS106 using a clonogenic assay. These results proved that TAS106 enhanced not only apoptosis but also reproductive cell death in X-irradiated tumor cells regardless of TP53 status and cell type.

TAS106 Enhances Caspase-Dependent Apoptosis and Reduces Accumulation of G$_2$/M Fraction in X-Irradiated Tumor Cells

To study the relationship between the radiation-induced cell cycle checkpoint and the induction of apoptosis by TAS106, flow cytometric analysis was performed. When cells were exposed to 20 Gy of X rays and incubated for 24 h for MKN45 and 48 h for MKN28, respectively, the flow cytometric profiles of both cell lines showed marked increases in the G$_2$/M fraction (57% for MKN45 and 78% for MKN28) but not in the sub-G$_1$ fractions (c in Fig. 2A and B), suggesting the occurrence of radiation-induced G$_2$/M arrest without apoptosis. As shown in (d) in Fig. 2A and B, the treatment with X rays + TAS106 resulted in decreases in the G$_2$/M fractions (28% for MKN45 and 44% for MKN28) and increases in the sub-G$_1$ fractions (16% for MKN45 and 28% for MKN28). The treatment of cells with TAS106 alone induced accumulation of the G$_1$ fraction but not an increase in the sub-G$_1$ fraction (b in Fig. 2A and B). Observation of the time course of the cell cycle further confirmed that X irradiation alone induced an increase of the G$_2$/M fraction and TAS106 enhanced radiation-induced apoptosis (sub-G$_1$ fraction) and reduced the radiation-induced increase of the G$_2$/M fraction (e–g in Fig. 2A and B). A similar reduction of the G$_2$/M fraction and enhancement of the radiation-induced apoptosis by treatment with TAS106 were also observed in irradiated Colon26 cells (data not shown).
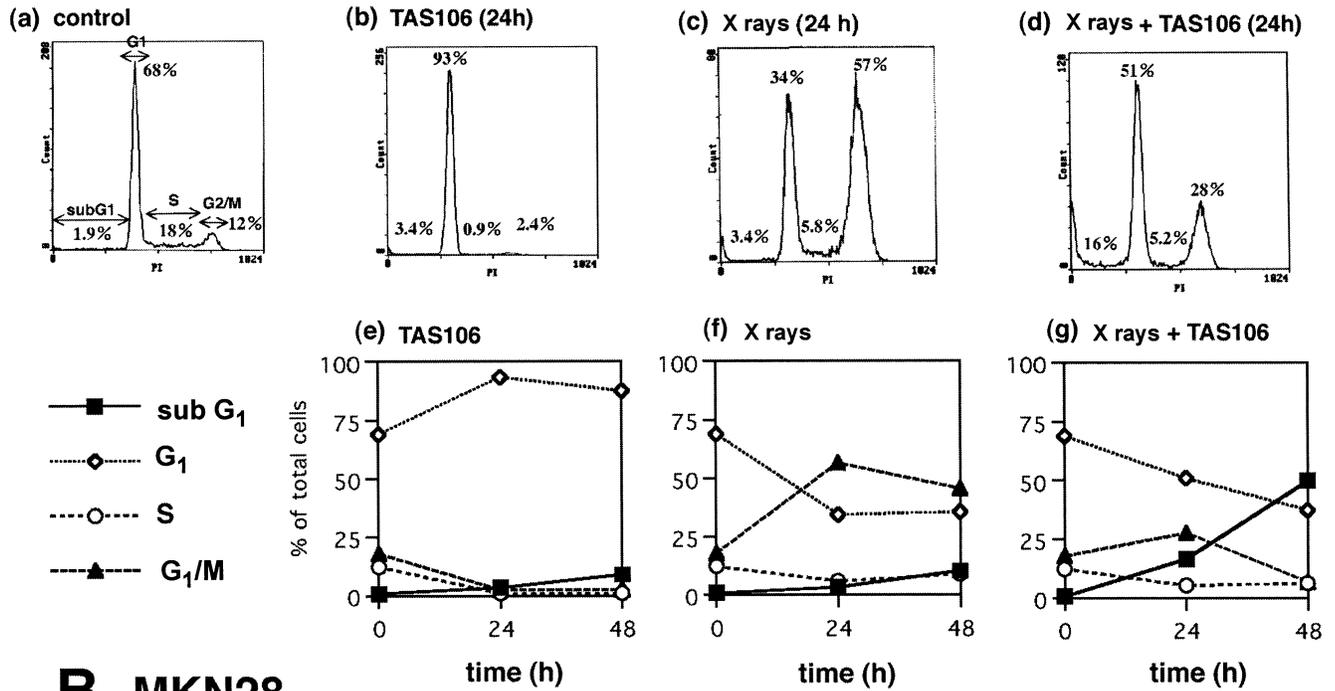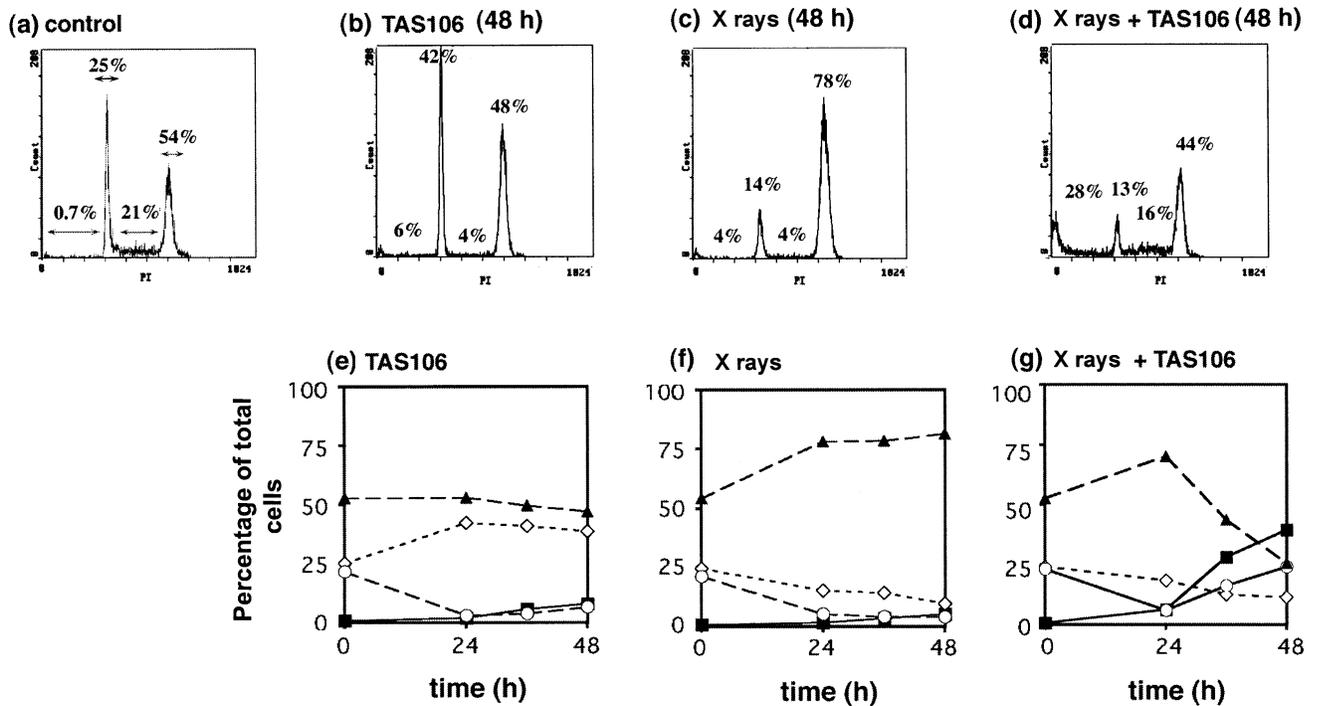
FIG. 2. Cell cycle analysis of MKN45 and MKN28 cells treated with TAS106 and/or X rays. Panel A: MKN45 cells treated with TAS106, X rays and X rays + TAS106 were incubated at 37°C for 24 and 48 h. Panel B: MKN28 cells treated with TAS106, 20 Gy of X rays or X rays + TAS106 were incubated at 37°C for 24 and 48 h. After incubation, cells were collected, fixed and stained with propidium iodide. The DNA content was measured by flow cytometry. Ten thousand events were captured for each treatment. Typical histograms of MKN45 (panel A) and MKN28 (panel B) cells from untreated control, TAS106, X rays and X rays + TAS106 are shown in (a) to (d), respectively. Time courses of cell cycle of cells exposed to TAS106, X rays or X rays + TAS106 are shown in (e), (f) and (g), respectively. (■) sub-G_1 fraction, ($\diamond$) G_1 fraction, (○) S fraction, (▲) G_2/M fraction.
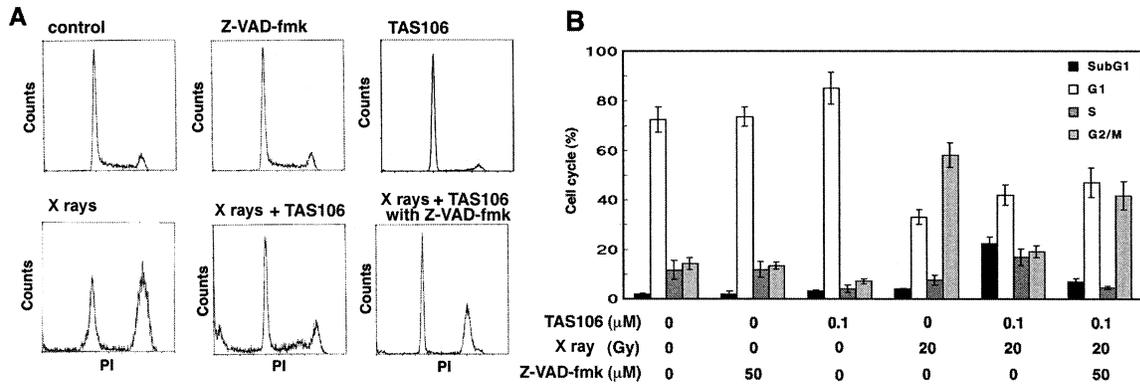
FIG. 3. Effects of a caspase inhibitor, Z-VAD-fmk, on cell cycle of MKN45 cells exposed to X rays + TAS106. Panel A: Cells were exposed to Z-VAD-fmk, TAS106, X rays, X rays + TAS106 or X rays + TAS106 with Z-VAD-fmk, and incubated at 37°C for 24 h. Cells were collected and stained with propidium iodide after fixation. The DNA content was measured by flow cytometry. Data concerning the cell cycle obtained from three independent experiments are summarized in panel B. Columns and bars represent means ± SEM.

We next examined the effect of a caspase inhibitor, Z-VAD-fmk, on apoptosis in MKN45 cells treated with X rays and/or TAS106. Figure 3 shows that the G_2/M fraction was markedly increased by treatment with X rays but not by that with Z-VAD-fmk or TAS106 in comparison with the control. Treatment with X rays + TAS106 significantly reduced the G_2/M fraction and increased the sub-G_1 fraction in comparison with the cells treated with X rays alone. The presence of Z-VAD-fmk significantly inhibited the formation of the sub-G_1 fraction and increased the G_2/M fraction in cells exposed to X rays + TAS106. Furthermore, using Ac-DEVD-MCA, a fluorescent caspase 3 substrate, we observed caspase 3 activation in the cell lysate at 36 h after X rays + TAS106 but not after X rays or TAS106 alone (data not shown). These results mean that caspase activation induced by X rays + TAS106 is involved in apoptosis.

TAS106 Attenuates Expression of Phospho-Cdc2, Cyclin B1 and Wee1 in MKN45 Cells

In cells exposed to X rays, the phosphorylation of Cdc2 and the accumulation of cyclin B1 are known to be important signals for the G_2/M checkpoint after DNA damage. In MKN45 cells X irradiation induced the accumulation of phospho-Cdc2 and cyclin B1 (left panel in Fig. 4A) and a slight increase in the histone H1 kinase activity of Cdc2 (second lane in Fig. 4B). Treatment with TAS106 not only suppressed basal expression of phospho-Cdc2, cyclin B1 and Wee1 (middle panel in Fig. 4A) but also abrogated X-ray-induced increases of expression of phospho-Cdc2 and cyclin B1 (right panel in Fig. 4A). The histone H1 kinase activity of Cdc2 was also remarkably inhibited by treatment with TAS106 in comparison with that after X rays alone (third and fourth lanes in Fig. 4B). These results indicate that TAS106 inhibits expression of cell cycle control protein during the radiation-induced G_2/M checkpoint.

Down-regulation of Expression of Survivin in MKN45, MKN28 and Colon26 Cells Treated with TAS106

To analyze the molecules responsible for X-ray-induced caspase-dependent apoptosis in the presence of TAS106,

the expression of anti-apoptosis proteins was examined in MKN45, MKN28 and Colon26 cells treated with TAS106. The results of Western blotting for survivin, Bcl2 and $BclX_L$ are shown in Fig. 5. In all cell lines, the expression of survivin was markedly suppressed by the treatment with TAS106 alone or X rays + TAS106. Large amounts of Bcl2 and $BclX_L$ protein existed in MKN45 cells treated with TAS106 alone or X rays + TAS106. However, for MKN28 and Colon26 cells, the expression of survivin and Bcl2 was suppressed markedly in cells treated with TAS106 alone or X rays + TAS106, but expression of $BclX_L$ was not changed by these treatments.

Transient Overexpression of Wild-Type Survivin Inhibits Apoptosis Induced by X Rays + TAS106

To clarify whether the down-regulation of survivin during the X-ray-induced G_2/M checkpoint was responsible for the induction of caspase-dependent apoptosis, we examined the effect of overexpression of wild-type survivin on radiation-induced cell death in the presence and absence of TAS106. The amount of survivin in cells transfected with pCIneo survivin was increased considerably in comparison with that of untransfected or mock-transfected cells as confirmed by Western blotting (Fig. 6A). Figure 6B shows the X-ray + TAS106-induced apoptosis in MKN45 cells overexpressing survivin. The cells with morphological changes indicative of apoptosis were scored GFP-positive cells, which contained pCIneo survivin. The induction of apoptosis by X rays + TAS106 was significantly inhibited by transfection of pCIneo survivin. These results strongly suggested that down-regulation of survivin by TAS106 played an important role in the radiosensitization of apoptosis in MKN45 cells. Next we examined effects of overexpression of survivin on abrogation of the G_2/M checkpoint by X-ray + TAS106 treatment. The sub-G_1 fraction was observed to be 24% in MKN45 cells at 36 h after transfection with pCIneo or pCIneo survivin [Fig. 6C (1) and C (2)]. When cells transfected with pCIneo were irradiated in the presence of TAS106 and incubated for 36 h, the sub-G_1 fraction
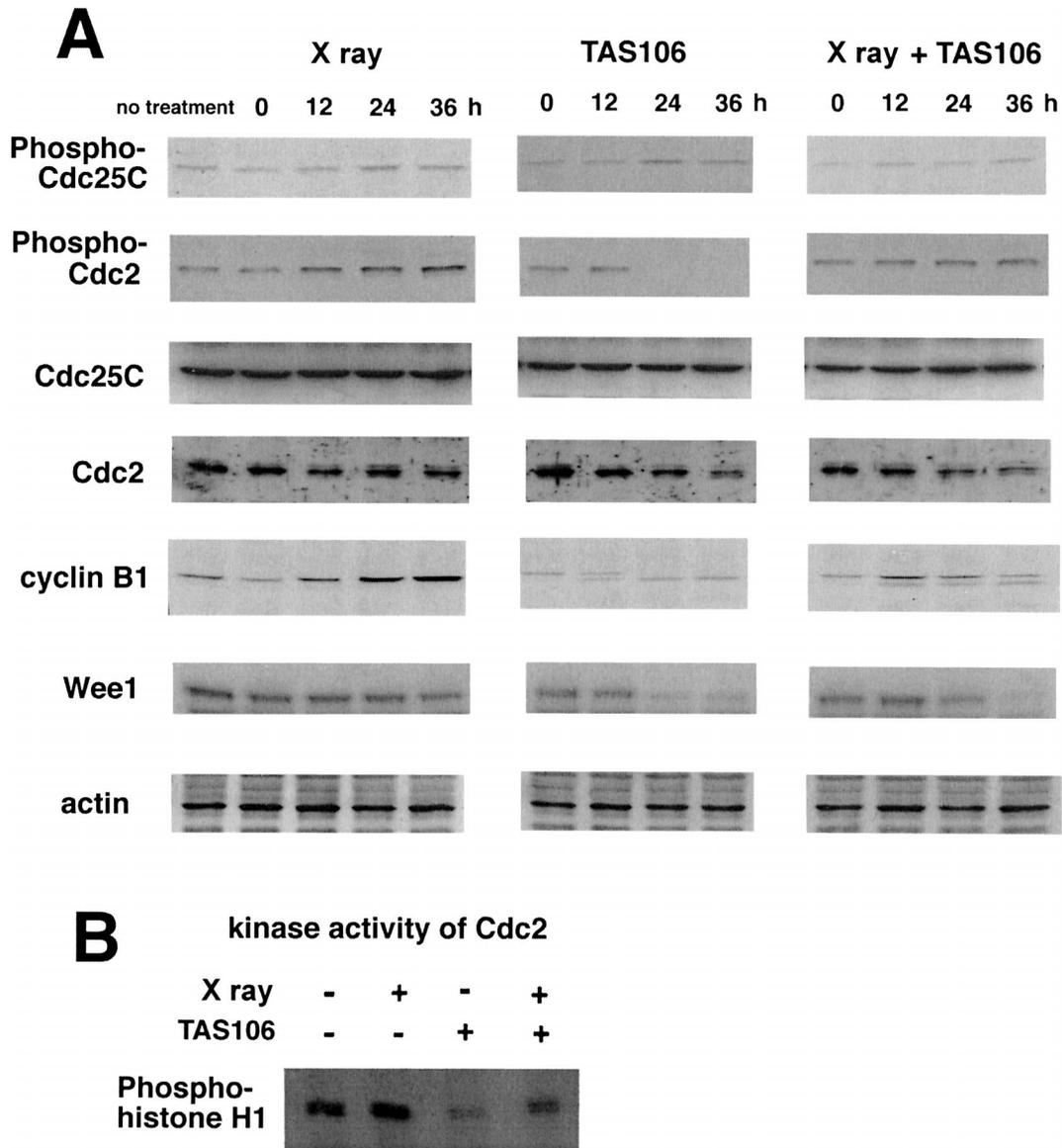
FIG. 4. TAS106 suppresses the expression of proteins related to the G$_2$/M checkpoint. MKN45 cells treated with X rays, TAS106 and X rays + TAS106 were incubated at 37°C for several hours. Panel A: Western blots of protein lysate (10 μg/lane) were prepared at the indicated times. Blots were probed with antibodies to phospho-Cdc25C, phospho-Cdc2, Cdc25, Cdc2, cyclin B1, Wee1 and actin, respectively. Panel B: Cdc2 was immunoprecipitated from MKN45 cells exposed to TAS106, X rays and X rays + TAS106 for 24 h. The reaction mixture of histone H1 and immunoprecipitated Cdc2 was incubated in the presence of [γ-^{32}P]ATP for 10 min at 30°C as described in the Materials and Methods. Phosphorylated histone H1 was subjected to 10% SDS-PAGE and visualized by autoradiography.

was 62% [Fig. 6C (3)]. Transfection with pCIneo survivin decreased X-ray + TAS106-induced apoptosis in the sub-G$_1$ fraction (42%) [Fig. 6C (4)]. Furthermore, the treatment with X rays + TAS106 induced reduction of the G$_2$/M fraction in the cells transfected with pCIneo, whereas transfection of pCIneo survivin significantly inhibited this X-ray + TAS106-induced decrease of the G$_2$/M fraction. Taken together, our present findings indicate that the loss of function of surviving caused by TAS106 is partly responsible for radiation-induced apoptosis during mitosis.

DISCUSSION

In radiosensitization of tumor cells and tissues, pharmacological agents with the ability to abrogate the G$_2$/M checkpoint have been of interest for many years. UCN-01 (2–6), an indolocarbazole (SB-218078) (7) and caffeine (8–10) have been shown to be able to abrogate the G$_2$/M checkpoint and enhance radiation-induced cytotoxicity. Recently, the preferential potentiation of radiation toxicity by UCN-01 was reported to cause apoptosis (6). The inhibition
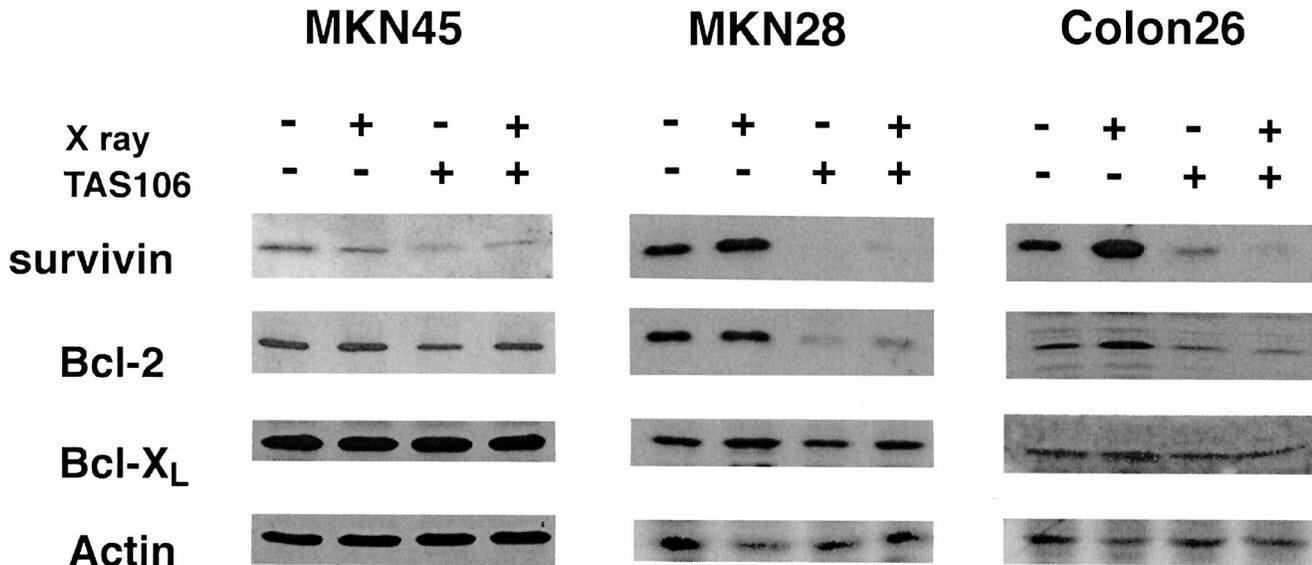
FIG. 5. Effects of TAS106 on the protein expression of survivin, Bcl2, BclX$_L$ and actin in MKN45, MKN28 and Colon26 cells exposed to X rays. Incubation in the presence of TAS106 was performed for 48 h for MKN28 cells, 24 h for MKN45 cells, and 24 h for Colon26 cells. Protein lysates were prepared from MKN45 (panel A), MKN28 (panel B) and Colon26 (panel C) cells exposed to TAS106, X rays or X rays + TAS106. Proteins of cell lysate (10 μg/lane) were separated using 15% SDS-PAGE and transferred to nitrocellulose membranes. Blot membranes were probed with antibodies to survivin, Bcl2, BclX$_L$ and actin as described in the Methods and Materials section, respectively.

of Chk1 by UCN-01 and SB-218078 and the inhibition of ATM and ATR by caffeine were proposed as mechanisms of the abrogation of the G$_2$/M checkpoint. Moreover, the down-regulation of Cdc2 and cyclin B1 by overexpression of double-stranded RNA-activated, serine/threonine protein kinase, PKR, was shown to induce apoptosis in CHO cells (*36*). In the present experiments, we found that a low concentration of a novel antitumor agent, TAS106, enhanced radiation-induced apoptosis and reproductive cell death, as shown in Fig. 1. Moreover, it seemed that this radiosensitization by TAS106 occurred in a TP53-independent manner because of observations obtained from the TP53-mutated cell line MKN28 (Fig. 1D and G and 2B). As shown in Fig. 3, an increase of the G$_2$/M fraction, but not the sub-G$_1$ fraction, was observed in MKN45 cells exposed to X rays only, and the treatment with TAS106 decreased this radiation-induced increase in the G$_2$/M fraction and enhanced the sub-G$_1$ fraction in MKN45 cells treated with X rays. As for expression of cell cycle proteins, TAS106 inhibited the Cdc2 kinase activity and reduced the protein expression of phospho-Cdc2, cyclin B1 and Wee1 in X-irradiated MKN45 cells (Fig. 5). These results may indicate an association between enhancement of radiation-induced cell death and abrogation of the G$_2$/M checkpoint by TAS106. However, further experiments will be necessary to clarify whether enhancement of radiation-induced cell death by TAS106 is caused by down-regulation of cell cycle proteins associated with the G$_2$/M progression and checkpoint.

Next, to search for the key molecule in X-ray + TAS106-induced apoptosis, we examined the effects of TAS106 on expression of anti-apoptosis proteins. Western

blotting showed that survivin was greatly suppressed by TAS106 in MKN45, MKN28 and Colon26 cells, and Bcl2 was moderately suppressed by TAS106 in MKN28 and Colon26 cells but not MKN45 cells. The expression of BclX$_L$ was hardly influenced by TAS106 in these three cell lines. The mechanism of the differential effect of TAS106 on the expression of anti-apoptosis proteins is unclear, but might be explained by the difference in the lifetime of mRNA in each protein, because TAS106 has been reported to be a global inhibitor for RNA synthesis in tumor cells (*28*). Raghavan *et al.* measured mRNA decay of 6000 expressed transcripts in T lymphocytes treated with actinomycin D by using microarray technology, and found half-lives from a few minutes ($T_{1/2} = 10$ min) to several hours ($T_{1/2} > 6$ h) and that numerous transcripts exhibited stimulus-dependent changes in their half-lives (*37*). In fact, we observed that the treatment with TAS106 for 24 h suppressed mRNA expression of survivin and Bcl2 but not that of BclX$_L$ and GAPDH in MKN45 cells (data not shown).

The expression of survivin was reported to be observable in embryonic and fetal organs but not in most terminally differentiated normal cells. By contrast, dramatic overexpression of survivin compared with normal tissues was demonstrated in tumors of the lung, breast, colon, esophagus, pancreas, bladder, stomach, uterus and ovary, as well as in melanoma, large cell non-Hodgkin's lymphoma, leukemia and neuroblastoma [see review by Altieli (*12*)], suggesting that alteration of survivin gene regulation occurs commonly during tumorigenesis. Overexpression of survivin was observed in 60 tumor cell lines on the NCI anti-cancer drug screening panel, demonstrating that transient overexpression of survivin inhibited etoposide-, Fas- and
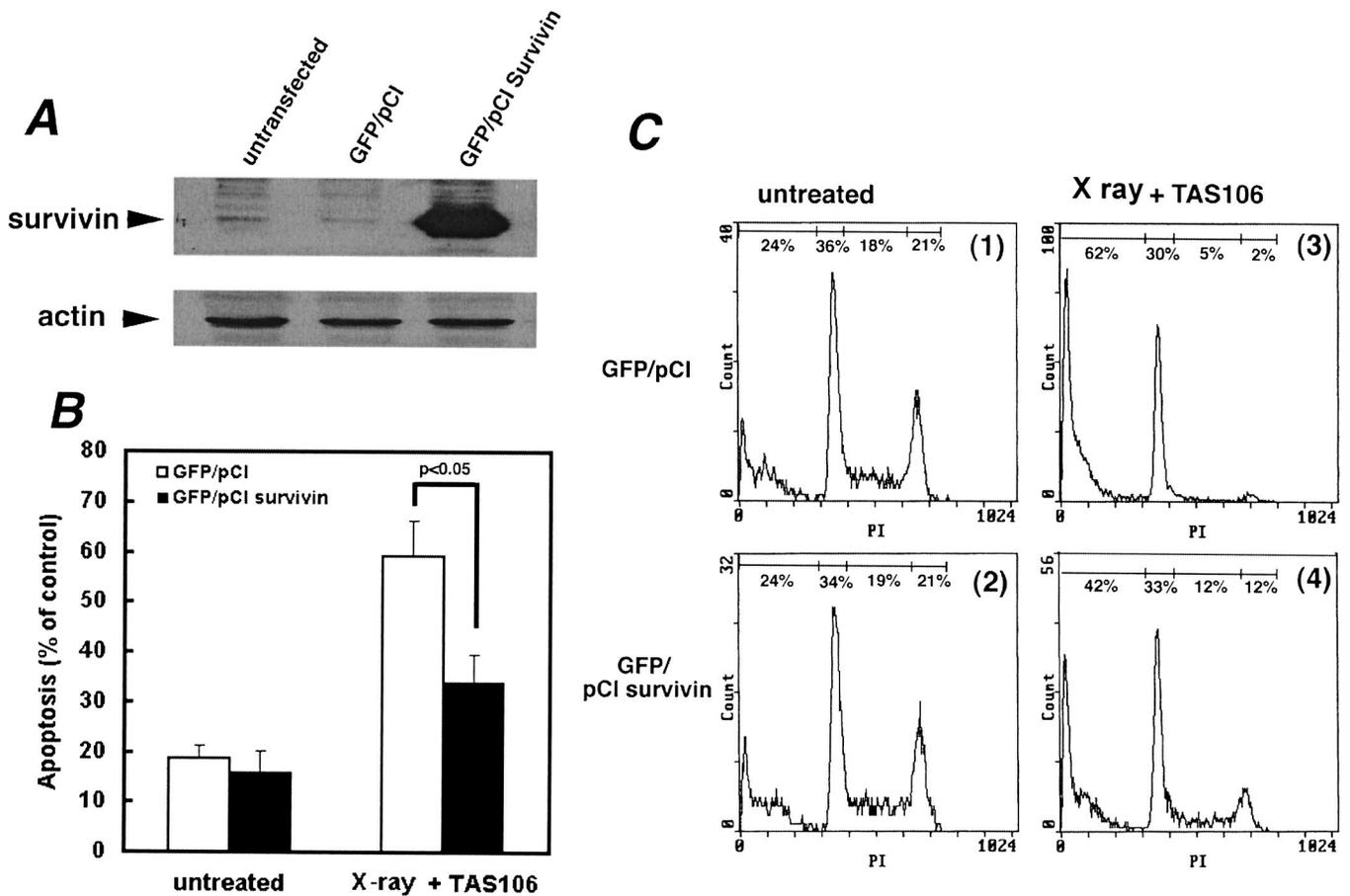
FIG. 6. Expression of wild-type survivin leads to inhibition of apoptosis induced in MKN45 cells treated with X rays + TAS106. MKN45 cells were transfected with pCIneo or pCIneo survivin, together with pQBI-GFP. Panel A: Western blotting of survivin expression and actin at 36 h after transfection. The MKN45 cells transfected with pCIneo or pCIneo survivin were incubated for 36 h after treatment with X rays + TAS106 and then fixed with 70% methanol. The cells were stained by propidium iodide. Panel A: The apoptotic cells among GFP-positive 500 cells were scored by a method similar to that described in the legend to Fig. 1A. Data are expressed as means ± SEM for three experiments. *$P < 0.05$ significant difference between apoptosis in the cells transfected with pCIneo and those with pCIneo survivin. Panel B: For analysis of the cell cycle, the GFP-positive cells were gated by flow cytometry and the cell cycle of the GFP-positive cells was examined.

Bax-induced caspase-dependent apoptosis (*16*). Yu *et al.* have demonstrated that five gastric cell lines, including MKN45 cells, and 34 of 50 human gastric cancer tissues (68%), highly express survivin mRNA (*38*). Several reports have demonstrated that radioresistance and chemoresistance in tumors are due to the overexpression of survivin (*13–15*). In this experiment, we found that the treatment with TAS106 efficiently induced down-regulation of survivin expression regardless of the type of tumor cell. This finding may be noteworthy for the application of TAS106 to tumor therapy.

O'Connor *et al.* showed that survivin overexpressed in cancer was physically associated with Cdc2 in the mitotic apparatus and phosphorylated on Thr34 by Cdc2-cyclin B1 *in vitro* and *in vivo* (*17*). This phosphorylation on Thr34 of survivin was required for the inhibition of both caspase-dependent and caspase-independent apoptosis of cells traversing mitosis (*17–20*). These data indicate that survivin serves as a mitotic substrate of Cdc2-cyclin B1 and that physical association of survivin with Cdc2-cyclin B1 is

necessary to preserve cell viability during cell division. In this study, it was shown that Cdc2 kinase activity was up-regulated by X-irradiated MKN45 cells and that TAS106 greatly suppressed this activity, as shown in Fig. 4B, suggesting that phosphorylated survivin, as an active form to inhibit the caspase cascade, might be reduced by TAS106-induced suppression of kinase activity of Cdc2. Furthermore, the demonstration that overexpression of survivin reduces X-ray + TAS106-induced apoptosis implies that loss of function of survivin is an important factor for radiosensitization in the induction of apoptosis in the G_2/M phase by TAS106.

RNA synthesis and protein synthesis inhibitors such as actinomycin D (Act D) (*39*) and cycloheximide (CHX) (*40*) are well known as strong inducers of apoptosis, and treatment of cells with Act D or CHX is reported to suppress expression of survivin (*24, 39*). We observed that Act D down-regulated survivin expression and radiosensitized MKN45 cells in a fashion similar to that of TAS106 (data not shown). However, general RNA or protein synthesis

inhibitors cannot be used for clinical application in tumor treatment because of their nonspecificity with regard to cell type. Matsuda and colleagues have demonstrated that TAS106 is phosphorylated by intracellular uridine/cytidine kinase 2 (*41, 42*), which has high activity in tumor cells relative to normal cells (*43*). This phosphorylated TAS106 (ECTP) gained high cytotoxicity for tumor cells through its inhibition of RNA synthesis. Thus TAS106 appears to have an advantage in specificity for tumor therapy. In this experiment, treatment with low doses of TAS106 induced abrogation of the X-ray-induced G_2/M checkpoint and the down-regulation of the anti-apoptosis molecule survivin and then enhanced not only apoptosis but also reproductive cell death. It is well known that apoptotic cells, relative to necrotic cells, are easily eliminated from normal tissue by phagocytosis without severe inflammatory responses. Therefore, the manipulation of the survivin/caspase pathway by using the combination of a low concentration of TAS106 and radiation may facilitate not only cell death but also elimination of cancer cells *in vivo*.

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