Cyclosporin-A Enhances Docetaxel-Induced Apoptosis through Inhibition of Nuclear Factor-κB Activation in Human Gastric Carcinoma Cells

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ABSTRACT

Purpose: **We sought to determine whether cyclosporin-A (CsA) enhances docetaxel [Taxotere (TXT)]-induced apoptosis in human gastric carcinoma cells, and, if so, to determine the relation between this apoptosis and nuclear factor-κB (NF-κB) activation.**

Experimental Design: **Two human gastric carcinoma cell lines (GCTM-1 and MK-1), a human embryonic pulmonary fibroblast cell line, and human umbilical vein endothelial cells were used as drug targets. Apoptotic cell death was verified morphologically by nuclear fragmentation assay with Hoechst staining. Electrophoretic mobility shift assays were performed to check for nuclear translocation of NF-κB. The therapeutic effects of a combination of TXT and CsA were assessed in a mouse peritoneal dissemination model.**

Results: **A combination of CsA (5 μM) and TXT (10 nM) significantly enhanced apoptotic cell death in both carcinoma cell lines but not in nonmalignant cell lines in comparison with the single-agent treatment alone. This effect was not related to drug uptake, efflux, or MDR1 expression. These effects were also observed in freshly obtained TXT-resistant gastric carcinoma cells isolated from a patient with malignant ascites. TXT alone induced NF-κB activation in both carcinoma cell types, and this activation was suppressed by CsA. A combination of TXT and NF-κB decoy, a well-known NF-κB inhibitor, also enhanced apoptotic cell death in the carcinoma cells. A combination of CsA and TXT significantly suppressed peritoneal dissemination *in vivo* relative to the single-agent effect.**

Conclusions: **Treatment with CsA and TXT in combination may be an effective therapeutic strategy for patients with gastric carcinoma.**

INTRODUCTION

Gastric carcinoma is one of the leading causes of cancer mortality worldwide (1, 2). It is generally thought that adjuvant chemotherapy provides modest prolongation of survival in some cases (3). TXT[2] is used mainly as a second-line chemotherapeutic agent for gastric carcinoma (4). It is a unique anticancer agent that poisons mitotic spindles by stabilizing microtubules, thus inhibiting their depolymerization to free tubulin (5, 6). TXT, as well as other types of anticancer drugs, induces MDR in carcinoma cells (7–9). Thus, strategies to overcome TXT-induced MDR are needed.

Several chemoresistant mechanisms of cancer cells have been proposed, including inactivation of the response mechanism to apoptotic stimuli [*e.g.*, mutated or deleted p53 tumor suppressor gene (10, 11)], expression of the MDR gene (12), and production of an inhibitor of apoptosis [*e.g.*, Bcl-2 or IAP3 (13, 14)]. NF-κB is a transcription factor that is involved in the antiapoptosis pathway in cancer cells (15, 16). Recent studies have shown that NF-κB activation plays a role in the development of chemotherapy resistance of carcinoma cells (17). We have shown that NF-κB is constitutively activated in human gastric carcinoma tissues and suggested that NF-κB activation is related to tumor progression (18). Those findings lead us to hypothesize that carcinoma cells exposed to TXT may activate NF-κB to escape from TXT-induced apoptosis and that NF-κB inhibitors may enhance TXT-induced apoptosis of carcinoma cells.

CsA is a clinically important immunosuppressive drug that is widely used to prevent graft rejection after organ or bone marrow transplantation (19, 20). CsA binds specifically to cyclophilins. The complexes formed by CsA and cyclophilins target and inhibit the Ca^{2+}- and calmodulin-dependent protein phosphatase calcineurin (21). Calcineurin plays a pivotal role in relaying membrane-associated signals to the nucleus. Therefore, CsA inhibits transcriptional activity mediated by key transcription factors, such as the

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[2] The abbreviations used are: TXT, docetaxel (Taxotere); NF-κB, nuclear factor κB; CsA, cyclosporin-A; EMSA, electrophoretic mobility shift assay; ODN, oligodeoxynucleotide; HUVEC, human umbilical vein endothelial cell; zVAD-FMK, benzyloxycarbonyl-valyl-L-alanyl-L-aspartyl-fluoromethylketone; RT-PCR, reverse transcription-PCR; FACS, fluorescence-activated cell-sorting; HPLC, high-performance liquid chromatography; MDR, multidrug resistance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

nuclear factor of activated T cells and NF-κB (22). It has been shown that CsA is a NF-κB inhibitor (23).

In this study, we examined whether TXT can simultaneously activate the apoptosis pathway and, through NF-κB activation, the antiapoptosis pathway and whether CsA enhances the TXT-induced apoptosis pathway via inhibition of NF-κB activation. We concluded that treatment with a combination of TXT and CsA may be an effective therapeutic strategy for gastric carcinoma.

MATERIALS AND METHODS

Cell Culture. Two human gastric adenocarcinoma cell lines, GCTM-1 and MK-1, were established in our laboratory from the ascites of cancer patients with peritoneal dissemination (24). The cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Life Technologies, Inc.) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C. Fresh carcinoma cells were prepared from the ascitic fluid of a patient with gastric carcinoma that had progressed despite TXT-based chemotherapy. Carcinoma cells were enriched by gradient sedimentation with lymphocyte separation medium (Litton Bionetics, Kensington, MD). The purity of carcinoma cells was >90%. Peripheral blood mononuclear cells from the blood of a healthy volunteer were also prepared by gradient sedimentation with lymphocyte separation medium. HUVECs were prepared as described previously (25). TIG-1-20 (JCRB0501), a human embryonic pulmonary fibroblast cell line, was obtained from Human Science Research and Resource Bank (Osaka, Japan). These three kinds of cells were used as normal control cells. We never failed to obtain informed consent from each patient before those preparations (fresh carcinoma cells, peripheral blood mononuclear cells, and HUVECs).

Reagents. CsA (Sandimmun; Sandoz, Tokyo, Japan) was diluted in absolute ethanol (stock solution, 50 mg/ml). TXT was purchased from Aventis Pharma Ltd. (Tokyo, Japan). EMSA ODNs were obtained from Promega (Madison, WI). All other ODNs were synthesized by Eurogenetec (Brussels, Belgium). ODNs for transduction experiments were double stranded and phosphorothioate modified at both the 5′ and 3′ ends. Sense strand ODNs were fluorescein labeled at the 3′ ends. The sequence of the NF-κB decoy sense strand was 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and contained both the specific p50 (GGGAC) and p65 (TTCC) κB binding sites (26). A nonsense sequence was used as scrambled ODN (5′-TTGCCGTACCTGACTTAGCCGT-3′; Ref. 27). Cationic liposomes were generated from Tfx-50 Reagent (Promega) and consisted of a mixture of the synthetic cationic lipid molecule *N*,*N*,*N*′,*N*′-tetramethyl-*N*,*N*′-bis(2-hydroxyethyl)-2,3,-dioleoyloxy-1,4-butanediaminium iodide and the fusogenic lipid L-dioleoyl phosphatidylethanolamine. Liposomes were used according to the supplier's instructions (27, 28).

Cell Transduction. DNA-liposome complexes were prepared just before use and added to the cells in a 2:1 liposome: DNA charge ratio (28). GCTM-1 cells were pretreated with either liposomes containing 0.5 μM ODN (NF-κB decoy ODN or scrambled ODN) or vehicle in serum-free RPMI 1640 for 30 min at 37°C. After one wash with PBS, GCTM-1 cells were stimulated at 37°C with 10 nM TXT and/or 5 μM CsA in RPMI 1640 supplemented with 10% FCS.

Apoptosis Assay. Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bis-benzimide stain (Hoechst 33342; Molecular Probes, Inc., Eugene, OR). Briefly, GCTM-1 cells (1×10^4 cells/well) were grown in flat-bottomed 96-well plates and cultured in the presence or absence of TXT (10 nM) with CsA (5 μM) for 3–24 h at 37°C and then stained with Hoechst 33342. Cells were observed with a fluorescence microscope, and nuclei that showed apoptotic chromatin changes were counted. The mean number of apoptotic nuclei in three fields (×200) was calculated. Cells with condensed or fragmented nuclei were considered apoptotic. Apoptosis was expressed as the percentage of all cells that were apoptotic.

Caspase Inhibitor. Caspase inhibitor zVAD-FMK was purchased from Enzyme Systems Products (Dublin, CA) and dissolved in DMSO at a stock concentration of 10 mM. GCTM-1 or MK-1 cells (1×10^4) were grown in 96-well plates and treated at 37°C for 18 h with a mixture of CsA and TXT with or without zVAD-FMK (10 μM).

Cytoplasmic or Nuclear Extraction. Cytoplasmic or nuclear extraction was performed in a cold room at 4°C according to the reagent manufacturer's instructions. Briefly, cells (5×10^5) incubated at 37°C in the presence or absence of CsA and TXT for 3 h were washed three times with ice-cold PBS and suspended in 50 μl of Cytoplasmic Extraction Reagent I (CERI; Pierce, Rockford, IL). The samples were incubated on ice for 10 min, and 2.8 μl of CERII (Pierce) were added to them. After incubation on ice for 1 min, samples were centrifuged for 5 min at maximum speed (16,000 × *g*) in a microcentrifuge. The supernatants were collected as cytoplasmic extracts, and the protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The nuclear pellets were resuspended in 25 μl of Nuclear Extraction Reagent (NER; Pierce) and incubated on ice for 40 min. After centrifugation for 10 min at maximum speed (16,000 × *g*) in a microcentrifuge, the supernatants were collected as nuclear extracts, and the protein concentrations were determined by Bio-Rad Protein Assay. Both extracts were stored at −80°C.

EMSA. To determine NF-κB activity, we performed EMSA as reported previously (29). Nuclear proteins (5 μg) were incubated for 30 min at 37°C with binding buffer [60 mM HEPES (pH 7.5), 180 mM KCl, 15 mM $MgCl_2$, 0.6 mM EDTA, and 24% glycerol], poly(deoxyinosinic-deoxycytidylic acid), and ^{32}P-labeled double-stranded oligonucleotide containing the binding motif of NF-κB (Promega). The sequence of the double-stranded oligomer used for EMSA was 5′-AGTTGAGGGGACTTTCCCAGGC-3′. The reaction mixtures were loaded onto a 4% polyacrylamide gel and electrophoresed with a running buffer of 0.25× Tris-borate EDTA. After the gel was dried, the DNA-protein complexes were visualized by autoradiography.

Western Blot Analysis. The protein lysate (40 μg) after each digestion was applied to a 15% SDS-polyacrylamide gel. The primary antibodies for caspase-3 (rabbit polyclonal antihuman caspase-3 IgG; Santa Cruz Biotechnology) and β-actin (rabbit antiactin IgG; Biomedical Technologies Inc., Stoughton, MA) were incubated with the blot at room temperature for 1 h at a dilution of 1:200. The secondary antibody (FITC-conjugated antirabbit IgG;

Santa Cruz Biotechnology) was incubated with the blot at room temperature at a dilution of 1:200 for 1 h. Visualization was performed with a Molecular Imager FX (Bio-Rad Laboratories) using the NIH Image program (Version 1.62; NIH Division of Computer Research and Technology, Bethesda, MD) on a Macintosh personal computer (Apple Computer, Inc., Cupertino, CA).

RT-PCR. Total RNA was extracted from carcinoma cells by the guanidinium thiocyanate-phenol-chloroform extraction method (30). Three μg of RNA were reverse transcribed to cDNA with the Superscript TM II RNase H-Reverse Transcriptase System (Life Technologies, Inc.). The PCR primers were 5′-CCACCCATGGCAAATTCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ for the GAPDH gene (31) and 5′-CAGGTGGTTGGAAGCTAACC-3′ and 5′-GAAGGCCAGAGCATAAGATGC-3′ for the human MDR type-1 (MDR1) gene (*mdr1*; Ref. 32). Amplification was performed in a DNA thermal cycler (Perkin-Elmer, Tokyo, Japan) according to the following protocol: (*a*) for *mdr1*, initial denaturation for 5 min at 95°C; 35 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 60°C, and polymerization for 45 s at 72°C; and final extension for 10 min at 72°C; and (*b*) for GAPDH, initial denaturation for 2 min at 95°C; 35 cycles of denaturation for 1 min at 95°C, primer annealing for 1 min at 58°C, and polymerization for 2 min at 72°C; and final extension for 15 min at 72°C. PCR products were separated on ethidium bromide-stained 1.5% agarose gels. Expected RT-PCR product sizes were 318 bp for *mdr1* and 593 bp for GAPDH.

FACS Analysis. To analyze expression of MDR1 in carcinoma cells, cells were incubated for 60 min on ice with purified mouse antihuman MDR1 IgG monoclonal antibody obtained from BD PharMingen (San Diego, CA). After a 1-h incubation, cells were washed twice with PBS containing 3% BSA (Sigma) and 0.1% NaN_3 [Sigma (referred to as FACS buffer)] and incubated in FACS buffer with the appropriate concentration of FITC-conjugated goat antimouse IgG antibody (BD PharMingen) for 45 min on ice. After a wash with FACS buffer, the fluorescence intensities of the gated cells populations were measured with a FACSCalibur flow cytometer and analyzed with CELLQuest software (Becton Dickinson).

HPLC Analysis. TXT concentration was determined by HPLC analysis as described previously (33). HPLC analysis was performed using a Simadzu Model LC-6A pump, Shimadzu SPD-6A UV detector (Kyoto, Japan) with 227 nm (34, 35), and a 4 × 125-mm Li-chrospher 100 RP-18 end-capped (4 μm) column (Merck, Darmstadt, Germany). TXT eluted with a mobile phase of 0.01 M phosphate buffer (pH 6.0):methanol (70:30, v/v).

Cellular uptake and efflux of TXT were determined by HPLC, with a modified version of the technique of den Hartigh *et al.* (36). GCTM-1 cells (1×10^6 cells/ml) were exposed to TXT in microtubes at 37°C. The resulting cell-drug incubation mixtures were further incubated from 15 to 120 min. They were then centrifuged at 1200 rpm for 10 min. Media containing the drug were carefully removed by aspiration, and the cells were rinsed with cold PBS. After rinsing, the cells were lysed with 500 μl of distilled water and scraped into a conical glass centrifuge tube. After 500 μl of 400 mM ammonium acetate buffer (pH 5) were added, the cells were extracted with 5 ml of diethyl ether. The mixture was centrifuged at 2000 rpm for 10 min. The upper, organic phase was collected and dried at 40°C under a stream of nitrogen. The sediment was resuspended in 20 μl of methanol and analyzed by HPLC.

Combined Therapeutic Efficacy in a Murine Model of Peritoneal Carcinomatosis. Colon-26 cells (2×10^5), a murine colon carcinoma cell line, were injected i.p. into female BALB/c mice (19–21 g; age, 7 weeks). The mice were housed in laminar flow cabinets under specific pathogen-free conditions in facilities approved by Kyushu University. After injection, mice were divided randomly into four groups (11 mice/group). On days 5 and 15, medium only (control group), 10 mg/kg TXT (TXT group), 5 mg/kg CsA (CsA group), or TXT plus CsA (combination group) was injected i.p. Mice were killed on day 21 after the injection of Colon-26 cells, and the numbers of disseminated implants in the peritoneal cavity were recorded.

Statistical Analysis. Results are expressed as mean ± SD. Statistical significance of the difference between group means was determined by Student's *t* test. All results with a *P* of <0.05 were considered statistically significant.

RESULTS

Effect of TXT and CsA in Combination on Apoptosis in Gastric Carcinoma Cells. We first observed the effects of TXT and/or CsA on apoptosis of GCTM-1 cells. TXT induced apoptosis in GCTM-1 cells in a dose-dependent manner (Fig. 1*A*). CsA alone did not induce apoptosis (Fig. 1*B*). When 5 μM CsA was combined with 5 or 10 nM TXT, apoptosis was induced to a greater extent than that seen with TXT only, and it increased in a time-dependent manner (Fig. 1, *C* and *D*). The presence of 5 μM CsA also increased 10 nM TXT-induced apoptosis in gastric carcinoma MK-1 cells. However, a combination of 5 μM CsA plus 10 nM TXT induced no detectable apoptosis in normal cells (Fig. 2). Although freshly isolated gastric carcinoma cells showed resistance to 1 μM TXT, the presence of 5 μM CsA significantly increased 10 nM TXT-induced apoptosis (Fig. 3).

Activation of Caspase-3 by TXT and CsA in Combination. zVAD-FMK, which is a broad-range caspase inhibitor, was applied to determine whether the activation of caspase is essential for nuclear fragmentation of gastric carcinoma cells treated with a combination of TXT and CsA. Apoptosis induced by a combination of TXT and CsA was blocked completely by 10 μM zVAD-FMK in GCTM-1 cells (Fig. 4*A*).

We then investigated whether caspase-3 is activated by exposure to TXT alone or only by a combination of TXT plus CsA in GCTM-1 cells. Activation of caspase-3 was determined by decreased expression of pro-caspase-3 based on Western blot analysis. The density of the pro-caspase-3 band was reduced more by exposure to CsA plus TXT than by exposure to TXT alone (Fig. 4*B*).

Activation of NF-κB by TXT and Suppression of TXT-Induced NF-κB Activation by CsA. Activation of NF-κB, which plays an important role in cell survival, was evaluated by EMSA. TXT induced NF-κB activation in GCTM-1 cells. Although CsA alone had no significant effect on NF-κB activation, CsA suppressed the NF-κB activation induced by TXT treatment (Fig. 5).

Enhancement of TXT-Induced Apoptosis with a NF-κB Decoy. To confirm the involvement of NF-κB in TXT-induced apoptosis, we examined the effect of a NF-κB decoy,

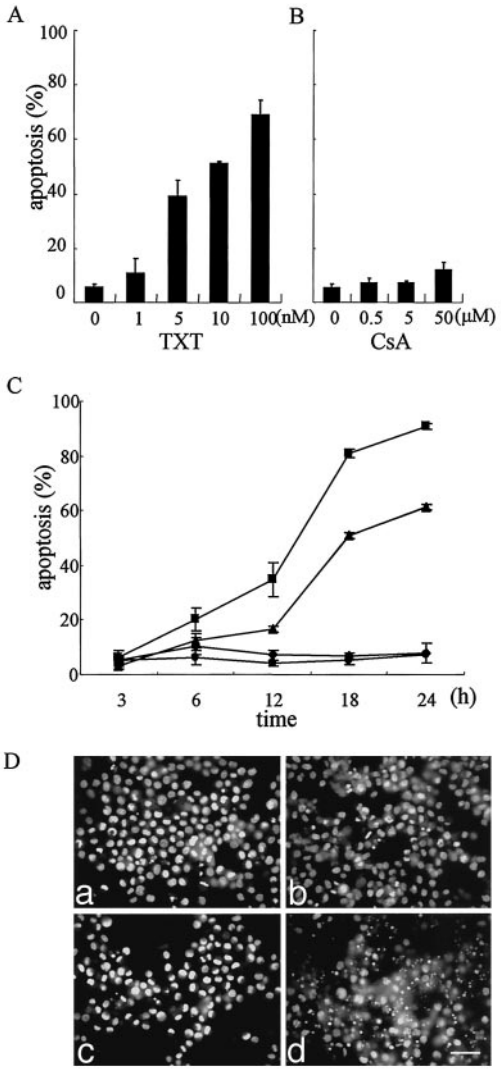

Fig. 1 Percentage of GCTM-1 cells with apoptosis. Values represent the mean ± SD of triplicate determinations. *A*, apoptosis of GCTM-1 cells after 24 h of treatment with various concentrations of TXT. *B*, apoptosis of GCTM-1 cells after 24 h of treatment with various concentrations of CsA. *C*, apoptosis of GCTM-1 cells after treatment with TXT and CsA (●, control; ◆, 5 μM CsA; ▲, 10 nM TXT; ■, 10 nM TXT plus 5 μM CsA) for various times. *D*, phase-contrast photomicrographs of GCTM-1 cells after 24 h of treatment with 10 nM TXT and 5 μM CsA. *a*, control; *b*, 10 nM TXT; *c*, 5 μM CsA; *d*, 10 nM TXT and 5 μM CsA. *Bar*, 50 μm.

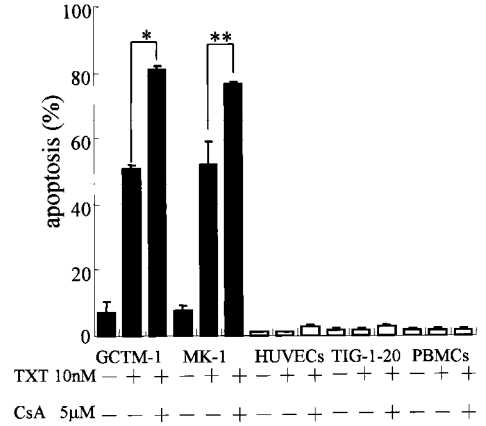

Fig. 2 Effects of CsA and TXT on the percentage of apoptotic cells in two gastric carcinoma cell lines and normal cells. Cells were incubated with a mixture of TXT (10 nM) and CsA (5 μM) for 24 h. + indicates the presence of agent; − indicates the absence of agent. Values represent the mean ± SD of triplicate determinations (*, $P < 0.01$; **, $P < 0.05$).

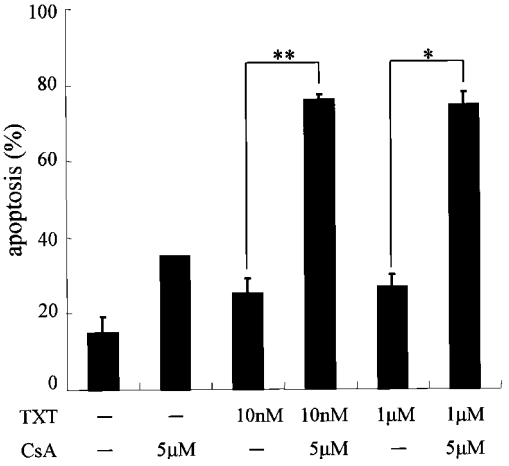

Fig. 3 Effects of CsA on TXT-induced apoptosis in freshly obtained gastric carcinoma cells. Concentrations of each agent present in the medium are specified on the *abscissa*. Values represent the mean ± SD of triplicate determinations (*, $P < 0.01$; **, $P < 0.05$).

which inhibits binding of nuclear NF-κB to DNA, on TXT-induced apoptosis in GCTM-1 cells. Although NF-κB decoy alone had no significant effect on induction of apoptosis in GCTM-1 cells, TXT combined with NF-κB decoy significantly increased TXT-induced apoptosis. NF-κB decoy did not change the apoptosis induced by TXT plus CsA (Fig. 6).

Evaluation of Expression of the MDR1 Gene in GCTM-1 Cells Treated with TXT by RT-PCR. To rule out the possibility that CsA is enhancing TXT through its effects on MDR-mediated drug efflux, we first determined the expression of MDR1 mRNA in GCTM-1 cells treated with TXT at various times. Fig. 7*A* shows that mdr1 mRNA was not detected in GCTM-1 cells treated with or without TXT. We also investigated the expression of MDR1 protein in those cells by FACS analysis and found that there was no expression of MDR1 protein in those cells (data not shown).

Effects of CsA on Uptake and Efflux of TXT in GCTM-1 Cells. We next examined whether CsA affects TXT accumulation in GCTM-1 cells (Fig. 7*B*). GCTM-1 cells were exposed to 1 μg/ml TXT with or without CsA (5 μM), and intracellular TXT levels were determined by HPLC at the times indicated between 15 and 120 min. There were no significant differences in the cellular levels of TXT between cells exposed to the mixture of TXT and CsA and those exposed to TXT alone. This finding indicated that CsA had no significant effects on the cellular accumulation of TXT in GCTM-1 cells.

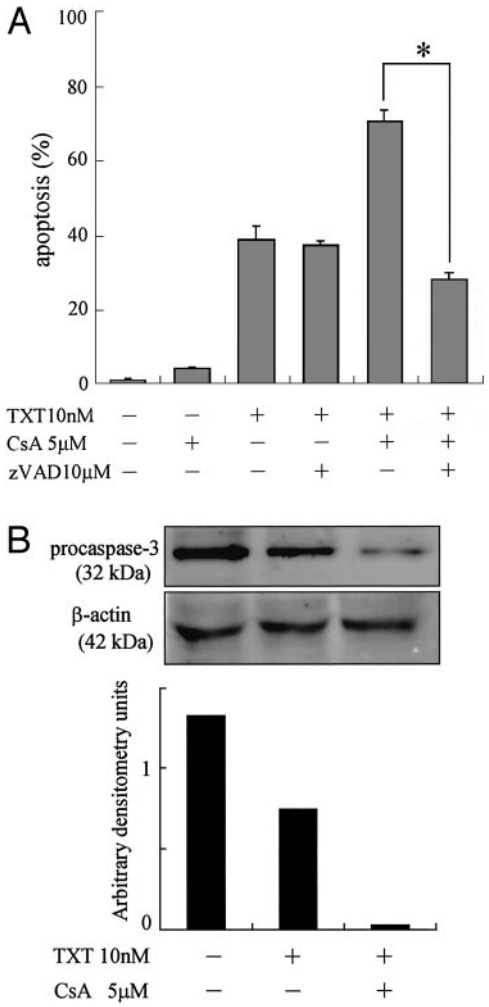

Fig. 4 *A*, the caspase inhibitor zVAD-FMK blocks TXT and CsA-induced apoptosis, but not TXT-induced apoptosis. GCTM-1 cells were incubated with a mixture of 10 nM TXT and 5 μM CsA with or without 10 μM zVAD-FMK for 18 h. Values represent the mean ± SD of triplicate determinations. *B*, activity of caspase-3 is reflected by the expression of pro-caspase-3. β-Actin served as an internal control. The densities of bands were quantified by NIH imaging densitometry. The data, from a single experiment, are representative of findings from three separate experiments and are presented as ratios to the corresponding β-actin.

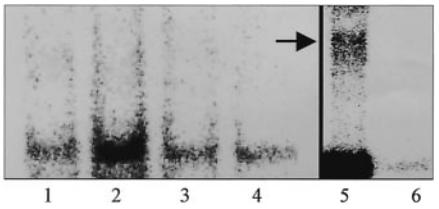

Fig. 5 Estimation of NF-κB DNA binding activity in GCTM-1 cells by EMSA. EMSAs were performed to identify the increased nuclear translocation of p65 in cells treated with TXT alone and the inhibition of this translocation in cells treated with TXT and CsA. *Lane 1*, control; *Lane 2*, 10 nM TXT; *Lane 3*, 5 μM CsA; *Lane 4*, 10 nM TXT and 5 μM CsA; *Lane 5*, plus p65; *Lane 6*, plus NF-κB ODN (×50).

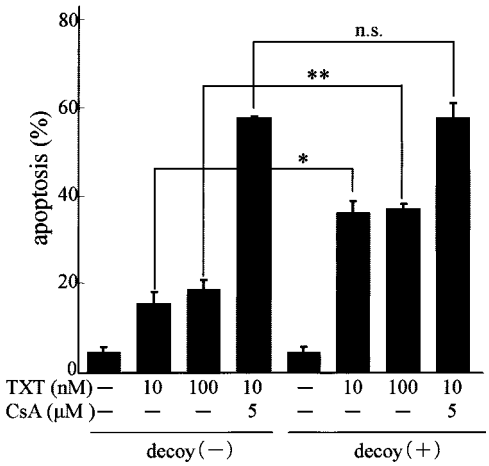

Fig. 6 NF-κB decoy enhanced TXT-induced apoptosis in GCTM-1 cells. GCTM-1 cells were either treated [*decoy(+)*] or not treated [*(decoy–)*] with NF-κB decoy (3 μg/10^6 cells) for 1 h before incubation with 10 nM TXT and 5 μM CsA for 18 h. Values represent the mean ± SD of triplicate determinations (*, $P < 0.01$; **, $P < 0.05$; *n.s.*, nonsignificant).

Next, the effects of CsA on TXT efflux from GCTM-1 cells were investigated (Fig. 7*C*). GCTM-1 cells were exposed to 1 μg/ml TXT for 1 h, rinsed twice in PBS, and then overlaid with fresh medium with or without CsA (5 μM). There were no significant differences in the cellular levels of TXT between the cells incubated with medium containing CsA and those incubated with CsA-free medium. This finding indicated that CsA had no significant effects on the efflux of TXT from GCTM-1 cells.

Suppression of Peritoneal Dissemination by i.p. Injection of TXT and CsA. We first examined whether a combination of TXT and CsA induces apoptosis in murine colonic carcinoma cells *in vitro*. TXT (10 nM) induced slight apoptosis in Colon-26 cells, and CsA alone (5 μM) had no effect. A combination of TXT and CsA induced marked apoptosis (Fig. 8*A*). Therapeutic experiments were then performed in a murine peritoneal dissemination model. Mice were sacrificed and examined macroscopically on day 21 after injection of Colon-26 cells. At least 28 disseminated nodules were noted in each of the control mice. TXT alone decreased the number of disseminated nodules ($P < 0.01$). CsA alone showed no significant effect on the formation of disseminated nodules. TXT combined with CsA significantly suppressed the formation of disseminated nodules compared with the effect of TXT alone ($P < 0.01$; Fig. 8*B*). Bloody ascites were found in 9 of 11 control mice but in only 1 of 11 mice treated with combination therapy ($P < 0.001$).

DISCUSSION

In the present study, we examined whether CsA could enhance TXT-induced apoptosis in cancer cells using several gastric carcinoma cell lines and freshly isolated gastric carcinoma cells. We demonstrated that CsA enhanced TXT-induced apoptosis in cancer cells but not in normal cells (such as

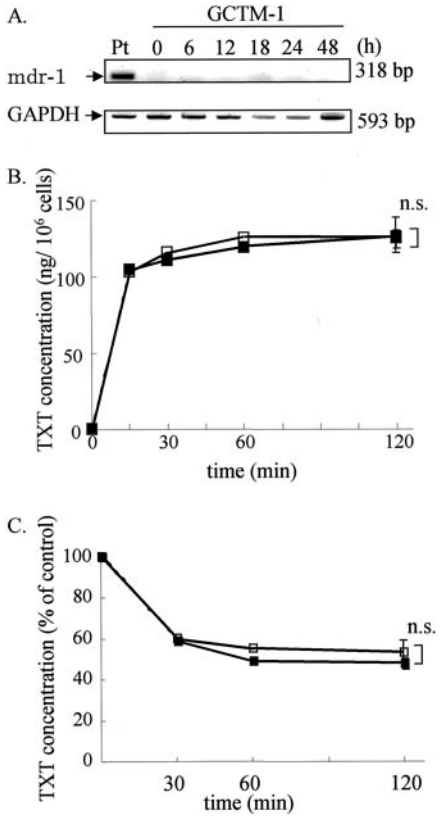

Fig. 7 *A,* expression of mdr1 mRNA on TXT-stimulated GCTM-1 cells and gastric carcinoma cells obtained from a patient who underwent TXT-based chemotherapy was examined by RT-PCR. TXT stimulation times are indicated. GAPDH mRNA served as an internal control. Gastric carcinoma cell mRNA obtained from a patient served as a positive control. *B,* effect of CsA on cellular accumulation of TXT in GCTM-1 cells. GCTM-1 cells were exposed to 1 μg/ml TXT with (■) or without (□) CsA (5 μM) for various times at 37°C. Values represent the mean ± SD of triplicate determinations after 120 min of treatment. *C,* effect of CsA on the efflux of TXT in GCTM-1 cells. GCTM-1 cells were exposed to 1 μg/ml TXT for 1 h at 37°C and then washed with cold PBS. The washed cells were resuspended in fresh medium with (■) or without (□) CsA (5 μM) and incubated for various times at 37°C. Values represent the mean ± SD of triplicate determinations after 120 min of treatment.

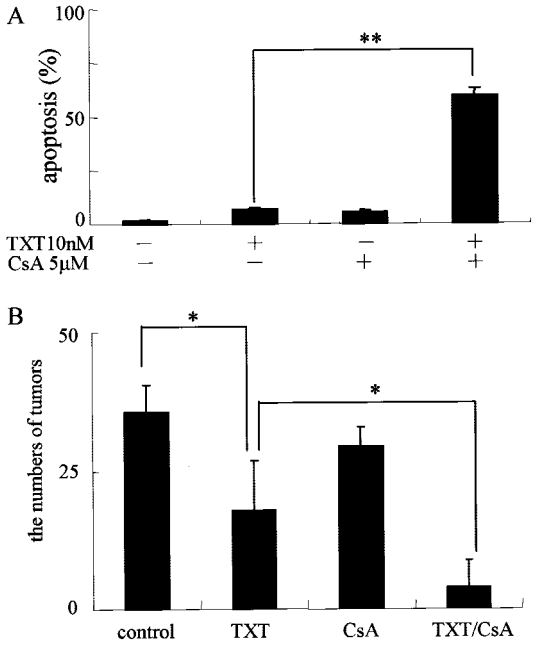

Fig. 8 Effect of CsA plus TXT on a murine tumor model. *A, in vitro* effect of CsA plus TXT on apoptosis of murine Colon-26 tumor cells incubated with 10 nM TXT and 5 μM CsA for 24 h. Values represent the mean ± SD of triplicate determinations (**, $P < 0.05$). *B,* number of tumors after each treatment by protocol (*, $P < 0.01$).

HUVECs and TIG-1-20 cells), and that this effect was due to the inhibition of NF-κB, an antiapoptotic transcription factor. We also showed that a combination of CsA and TXT was more effective in the inhibition of tumor development than each agent alone in mouse peritoneal tumor models.

We showed that CsA was also effective in enhancing TXT-induced apoptosis in freshly isolated gastric carcinoma cells. This is clinically very important. We encountered a patient with malignant ascites, who had received several chemotherapeutic agents including TXT and had a poor response consistent with multiple drug resistance. In fact, the fresh carcinoma cells obtained from the ascitic fluid were resistant to a high concentration of TXT (1 μM). However, a combination of 5 μM CsA plus TXT induced marked apoptosis in the fresh carcinoma cells, even when a low concentration of TXT (10 nM) was used. Fresh adenocarcinoma cells were prepared from three other gastric carcinoma patients. A combination of CsA and TXT also induced enhanced apoptosis in two of the three cases (data not shown). We suspect that CsA blocks the development of resistance to apoptosis induced by chemotherapeutic agents.

An activated form of NF-κB has been implicated in the development of resistance to chemotherapeutic agents (37). It has been shown that NF-κB inhibitors such as PS-341 enhance the anticancer effects of chemotherapeutic agents (38, 39), and it has been reported that TXT induced NF-κB activation in several types of malignant cells (40–42). Our data also showed that TXT induced NF-κB activation in gastric carcinoma cells. We hypothesized that CsA enhances TXT-induced apoptosis by inhibiting TXT-induced activation of NF-κB. This hypothesis was supported by our observations that NF-κB decoy, which could block the binding of NF-κB to DNA, enhanced TXT-induced apoptosis and that the NF-κB decoy had no effect on the apoptosis induced by a combination of TXT and CsA. From these observations, we strongly suggest that CsA enhances TXT-induced apoptosis mainly via inhibition of NF-κB activation.

Furthermore, we showed that CsA had no significant effects on the uptake and efflux of TXT in GCTM-1 cells. Recent studies have shown that CsA enhances the effects of anticancer drugs in leukemia cells through MDR1 blocking (43–45). It was recently demonstrated that MDR1 is one of the downstream genes of NF-κB (46–48). Therefore, MDR1 could be associated with CsA enhancement of TXT-induced apoptosis. In our study, however, TXT did not induce expression of MDR1 in GCTM-1 cells at the mRNA or protein level (P-glycoprotein). In addition, verapamil, a MDR inhibitor (49), had no effect on apoptosis or

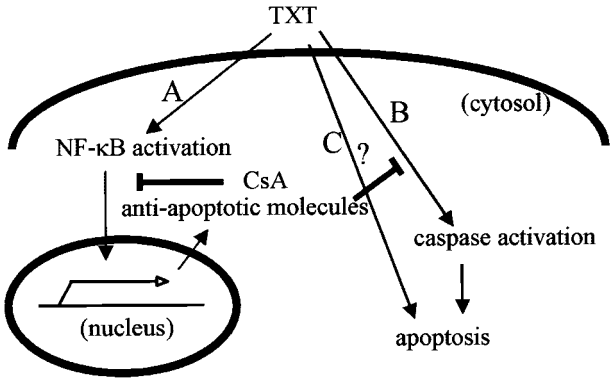

Fig. 9 A proposed mechanistic model of synergistic induction of apoptosis by TXT plus CsA. *A*, antiapoptosis pathway. *B*, caspase-dependent apoptosis pathway. *C*, caspase-independent apoptosis pathway.

NF-κB activation in GCTM-1 cells (data not shown). Collectively, these results suggested that CsA enhanced TXT-induced apoptosis mainly through a MDR1-independent antiapoptosis pathway in GCTM-1 cells. However, CsA enhanced uptake of TXT and suppressed its efflux in MDR-expressing KB-C1 cells (Ref. 50; data not shown). Additional studies need to be done to elucidate the relationship between CsA-mediated NF-κB activation and MDR gene.

Although enhanced apoptosis induced by a combination of CsA and TXT was blocked completely by 10 μM zVAD-FMK, apoptosis induced by TXT alone was not affected. These findings lead us to propose a mechanism for CsA/TXT-induced apoptosis (Fig. 9). When tumor cells are exposed to TXT alone, caspase-dependent and -independent apoptosis pathways and a CsA-sensitive antiapoptosis pathway are activated simultaneously (51). The CsA-sensitive antiapoptosis pathway, which is critically dependent on NF-κB, inhibits the caspase-dependent apoptosis pathway, so apoptosis is induced through the caspase-independent apoptosis pathway. When CsA is combined with TXT, the CsA-sensitive antiapoptosis pathway is blocked. As a result, TXT induces a high level of apoptosis through both the caspase-independent and -dependent pathways. However, the identity of the antiapoptosis substances acting in the CsA-sensitive antiapoptosis pathway is unknown. We propose that the antiapoptosis substance may be a target gene of NF-κB.

In our preliminary *in vivo* experiments, we showed that treatment with a combination of CsA and TXT caused a larger antitumor effect than treatment with CsA or TXT alone. Although the effect of the combination on survival rate or time has not yet been determined, the formation of bloody ascites was significantly reduced in the combination treatment group. A limitation of this *in vivo* experiment is that drug delivery and the timing of drug combination are insufficient. Additional studies are needed to clarify the *in vivo* effect of the combination.

CsA enhanced TXT-induced apoptosis in two human gastric carcinoma cells mainly through the inhibition of TXT-induced NF-κB activation. CsA enhancement of TXT-induced apoptosis was seen in some cases of fresh carcinoma cells and *in vivo* experiments. We believe that treatment with a combination of CsA and TXT will prove to be a useful therapeutic strategy for gastric cancer patients, especially for patients with MDR.

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