

## A Protein-bound Polysaccharide, PSK, Enhances Tumor Suppression Induced by Docetaxel in a Gastric Cancer Xenograft Model

AKIO YAMASAKI<sup>1</sup>, MOMOKO SHODA<sup>2</sup>, HIROKO IJIMA<sup>2</sup>, SHUNTARO NAGAI<sup>1</sup>, JUNJI WADA<sup>1</sup>, HIROYUKI SUZUKI<sup>1</sup>, NOBUHITO CHIKAZAWA<sup>1</sup>, TAKEHIKO TASAKA<sup>1</sup>, CHIZU KAMEDA<sup>1</sup>, HARUO TANAKA<sup>1</sup>, MIO IKEBE<sup>1</sup>, EI JO<sup>1</sup>, NORIHIRO SATO<sup>1</sup>, MASAFUMI NAKAMURA<sup>1</sup>, FUJIO SEKINE<sup>2</sup>, TAKASHI MORISAKI<sup>1,3</sup> and MITSUO KATANO<sup>1</sup>

<sup>1</sup>Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, Fukuoka; <sup>2</sup>Kureha Chemical Industry, Tokyo; <sup>3</sup>Yakuin CA Clinic, Fukuoka, Japan

**Abstract.** *Background:* We have reported previously that docetaxel (TXT) induces apoptosis and nuclear factor-kappaB (NF- $\kappa$ B) activation, and that blockade of NF- $\kappa$ B activation augments TXT-induced apoptosis in human gastric cancer cells. In addition, we have also shown that a protein-bound polysaccharide PSK enhances TXT-induced apoptosis through NF- $\kappa$ B inhibition in human pancreatic cancer cells. Based on these observations, in the present study the possibility that PSK could enhance TXT-mediated tumor suppression was examined in vivo and in vitro. *Materials and Methods:* A gastric cancer xenograft model was used to examine the enhanced TXT-mediated tumor suppression by PSK in vivo. The effects of PSK on proliferation and apoptosis induced by TXT in gastric cancer cells were evaluated in vitro using a human gastric cancer cell line, MK-1. The effect of PSK on increased TXT-induced invasion was also measured. *Results:* PSK enhanced TXT-mediated tumor suppression in vivo. Immunohistochemical analyses of the tumors revealed that TXT increased NF- $\kappa$ B activation in the tumors and this was suppressed by PSK. In the ex vivo experimental system, PSK enhanced the growth inhibition and apoptosis induced by TXT in the MK-1 cells and reduced the increased invasive ability induced by TXT. *Conclusion:* PSK enhanced TXT-induced tumor suppression in a gastric cancer xenograft model.

Gastric carcinoma is one of the leading causes of cancer mortality worldwide (1, 2). It has been generally considered that adjuvant chemotherapy provides modest prolongation of survival

*Correspondence to:* Professor Mitsuo Katano, Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81 926426941, Fax: +81 926426221, e-mail: mkatano@tumor.med.kyushu-u.ac.jp

*Key Words:* Gastric cancer, xenograft model, docetaxel, PSK.

in some cases. Docetaxel (TXT), a chemotherapeutic agent belonging to the taxane family, is a unique anticancer agent that disrupts mitotic spindles by stabilizing microtubules, thus inhibiting their depolymerization to free tubulin (3, 4). TXT is now used as a second-line chemotherapeutic agent for various types of cancer, including gastric cancer (5). However, TXT, as well as other types of anticancer drugs, induces multi-drug resistance (MDR) in cancer cells (6, 7). Therefore, therapeutic strategies to overcome TXT-induced MDR are needed.

Nuclear factor-kappaB (NF- $\kappa$ B) is a transcription factor involved in regulating multiple cellular activities such as cell proliferation and cytokine induction in many types of normal cells (8). It has also been shown that constitutive NF- $\kappa$ B activity plays an important role in the protection of tumor cells from apoptosis (9, 10). We have previously shown that NF- $\kappa$ B is constitutively activated in human gastric carcinoma tissue and suggested that NF- $\kappa$ B activation is related to tumor progression since a positive correlation of the grade of NF- $\kappa$ B activation with the depth of tumor invasion was observed (11). In addition, we have shown that TXT enhances NF- $\kappa$ B activation in gastric cancer cells and pancreatic cancer cells (12, 13). These findings led us to hypothesize that cancer cells exposed to TXT may escape from TXT-induced apoptosis through NF- $\kappa$ B activation. If so, blockade of NF- $\kappa$ B activation may enhance TXT-induced apoptosis in carcinoma cells.

PSK, a protein-bound polysaccharide, has been used as a nonspecific immunostimulant for treating cancer patients in Japan for more than 30 years (14). PSK possesses antitumor activity, such as the induction of apoptosis and suppression of angiogenesis (12, 15), and the combination of PSK with various chemotherapeutic agents has been tried as a new treatment regimen using animal models (16-20). Beneficial therapeutic effects have been shown in clinical studies for several types of tumors, including gastric cancer (21-23). Despite these interesting observations, the effect of PSK on

the modulation of chemotherapy-induced apoptosis has been little investigated and to improve the efficacy of combination therapy of PSK and chemotherapeutic agents, clarification of the antitumor mechanisms is needed.

In the present study, the possibility that PSK could enhance the antitumor effect of TXT was examined in a gastric cancer xenograft model.

## Materials and Methods

**Cells and reagents.** A human gastric adenocarcinoma cell line, MK-1, was established in our laboratory from ascites of cancer patients with peritoneal dissemination (24). The cells were maintained in RPMI-1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FCS; Life Technologies) and antibiotics (100 units/ml penicillin (Meijiseika, Tokyo, Japan) and 100 µg/ml streptomycin (Meijiseika)), referred to as complete culture medium, at 37°C, in 5% CO<sub>2</sub>. TXT was purchased from Rhone-Poulenc Rorer (Antony, France), and PSK was provided by Kureha Chemical Industry (Tokyo, Japan). The primary antibody used for immunohistochemistry was anti-NF-κB p65 (sc-109; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody for immunohistochemistry was purchased from Nichirei (Tokyo, Japan).

**Gastric cancer xenograft model.** All the animal experiments were approved by the Institutional Animal Care and Use Committee of Kureha Chemical Industry. MK-1 cells (10<sup>7</sup> cells) were injected subcutaneously into the flanks of 4-week-old female athymic BALB/C nude mice. Tumors became palpable within 5 days of tumor cell injection, after which the animals were randomized and assigned to different treatment groups (n=6 in each group). The animals were injected intraperitoneally with PSK (50 mg/kg) dissolved in phosphate-buffered saline solution (PBS) (PSK group) or PBS alone (control group), 3 times every week from 7 days after tumor cell injection. TXT (2.5 or 5 mg/kg) or vehicle was injected intravenously on days 14 and 21 after tumor cell injection. The mice were sacrificed and the tumors resected on day 49 after tumor injection. Tumor size was calculated using the following formula:  $\pi/6 \times \text{large diameter} \times (\text{small diameter})^2$ .

**Immunohistochemistry.** For immunostaining, frozen tumor sections (4 µm) were fixed in 10% formalin and embedded in paraffin. The slides were immersed in 3% H<sub>2</sub>O<sub>2</sub> in methanol and 10% normal goat or rabbit serum (Santa Cruz Biotechnology) for 30 min and each section was then incubated with the optimal concentration of primary antibody for 24-48 h at 4°C. After the sections were incubated with appropriate secondary antibodies, the immune complexes were detected with a combination of 3,3'-diaminobenzidine (40 mg/150 ml in PBS; Wako Pure Chemical Industries, Hyogo, Japan) and 0.06% hydrogen peroxide. The specimens were photographed with a digital camera attached to a microscope (BX51; Olympus Corp., Tokyo, Japan). Nuclear staining, which reflects nuclear translocation of p65, was considered a marker of NF-κB activation.

**Semiquantitative reverse transcription-PCR (RT-PCR).** The total RNA was extracted from the MK-1 cells with the guanidinium isothiocyanate-phenol-chloroform extraction method (25) and cDNA was synthesized by reverse-transcribing the total RNA with

ImProm-II Reverse Transcriptase (Promega Corp, Madison, WI, USA). Standard PCR reactions were completed using HotStarTaq DNA polymerase (Qiagen, Valencia, CA, USA).

The sequences of the primers used were matrix metalloproteinase-9 (MMP-9): forward, 5'-TGGGCTACGTGACCTATGACAT-3', reverse, 5'-GCCAGCCCACCTCCACTCCTC-3'; glyceraldehyde-3-phosphate-dehydrogenase (GAPDH): forward, 5'-CCACCCATGGCAAATTC ATGGCA-3', reverse, 5'-TCTAGACGGCAGGTCA GGTCCACC-3'. MMP-9 and GAPDH amplification conditions comprised an initial denaturation for 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C. Amplification of each gene was in the linear range. The PCR products were separated on ethidium bromide-stained 2% agarose gels. Semiquantitative analysis was conducted with a Molecular Imager Fx Pro (Bio-Rad, Hercules, CA, USA) to obtain the MMP-9/GAPDH ratios.

**Proliferation assay.** To examine the proliferation of the MK-1 cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (26) was performed. The cells were seeded in 96-well flat-bottomed microplates at a density of 2×10<sup>4</sup> cells/well in 200 µl of culture media containing different concentrations of TXT and PSK and incubated at 37°C for 24 h. Cell proliferation was assessed in triplicate.

**Detection of apoptosis.** Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with Hoechst 33342 (Wako, Osaka, Japan). Briefly, the cells were plated in 96-well plates (2×10<sup>5</sup> cells/well), treated with TXT, PSK or both for 24 h; stained with Hoechst 33342, and then observed under fluorescence microscopy. A total of 1,000 cells were observed in five randomly chosen fields.

**Electrophoretic mobility shift assay (EMSA).** The NF-κB activity in the nuclei isolated from MK-1 cells was determined by EMSA. Extraction of nuclear proteins and EMSA were carried out as described elsewhere (11). Briefly, 10 µg of nuclear proteins were incubated for 30 min at room temperature with binding buffer (20 mM HEPES-NaOH [pH 7.9], 2 mM EDTA, 100 mM NaCl, 10% glycerol, 0.2% NP-40), poly(deoxyinosinic-deoxycytidylic acid), and <sup>32</sup>P-labeled double-stranded oligonucleotide containing the NF-κB binding motif (Promega Corp). The sequence of the double-stranded oligomers used for EMSA was 5'-AGTTGAGGGGACTTTCCAGGC-3'. The reaction mixtures were loaded on a 5% polyacrylamide gel and electrophoresed with a running buffer of 5% Tris-borate EDTA. After the gel was dried, the DNA-protein complexes were visualized by autoradiography. The double-strand oligomer Oct-1 probe (Promega Corp) was used in the competitive studies. The sequence of the Oct-1 probe was 5'-TGTCGAATGCAAATCACTAGAA-3'.

**Matrigel invasion assay.** The invasiveness of the MK-1 cells was assessed based on the invasion of cells through Matrigel-coated transwell inserts, according to the method of Albini *et al.* (27) with some modifications. In brief, the upper surface of a filter (pore size, 8.0 µm; BD Biosciences, Heidelberg, Germany) was coated with basement membrane Matrigel (BD Biosciences) at a concentration of 2 mg/ml, and incubated at 4°C for 1 h; unbound material was aspirated. The cells were suspended in RPMI-1640 with 10% FCS containing the desired dose of reagents. Then, 0.8×10<sup>5</sup> cells were added to the upper chamber and incubated in a water-saturated 5% CO<sub>2</sub> atmosphere at 37°C for 16 h. After incubation, the filter was fixed with 70% ethanol and stained with Diff-Quik reagent

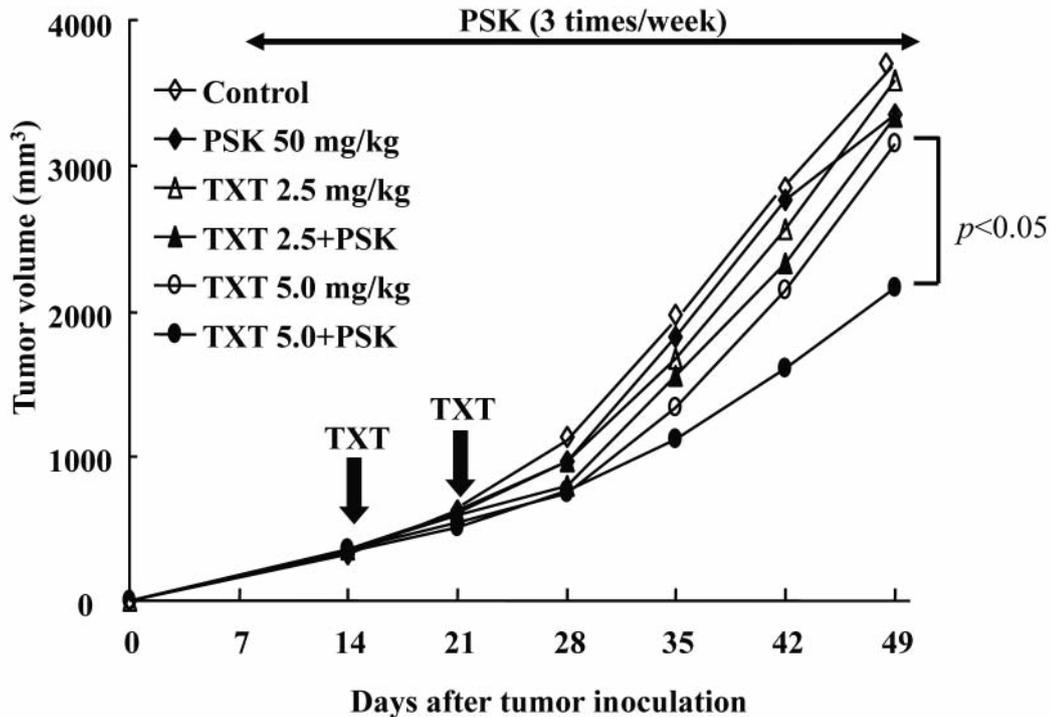


Figure 1. Effect of PSK on the antitumor activity of TXT in a gastric cancer xenograft model. BALB/c nude mice were injected with the MK-1 cells and from day 7 were injected with PBS (control) or PSK (50 mg/kg).

(International Reagents, Kobe, Japan). The cells on the upper surface were then completely removed by wiping with a cotton swab. The cells that had migrated from the upper to the lower side of the filter were counted under a light microscope (BX50; Olympus Corp.) at a magnification of  $\times 80$ . Tumor cell invasiveness was defined as the total number of cells in five randomly selected microscopic fields. Each experiment was performed in triplicate wells.

**Statistical analysis.** The Student *t*-test was used for statistical analysis. *P*-value less than 0.05 was considered significant.

## Results

**Effect of PSK on antitumor activity of TXT in a gastric cancer xenograft model.** Treatment with PSK or 2.5 mg/kg TXT alone did not affect the kinetics of tumor growth (Figure 1). Following treatment with 5 mg/kg TXT alone, the tumor size was slightly reduced when compared with that of the vehicle-treated control group. The tumor size was significantly reduced in mice treated with 5 mg/kg TXT + PSK. No mouse died during the observation period. Weight loss and skin abnormalities were not observed through the different treatment cycles.

**Effect of TXT and PSK on NF- $\kappa$ B activation in tumors.** Slight constitutive activation of NF- $\kappa$ B was detected in a small part of the tumor from the control mice (Figure 2A). PSK alone did not significantly affect NF- $\kappa$ B activation (Figure 2B). On

the other hand, TXT (5 mg/kg) alone remarkably enhanced NF- $\kappa$ B activation (Figure 2C). Importantly, PSK suppressed the enhanced TXT-induced NF- $\kappa$ B activation (Figure 2D).

**Effect of TXT and PSK on NF- $\kappa$ B activation in the MK-1 cells.** When the MK-1 cells were treated with TXT (1 nM) for 15 min, NF- $\kappa$ B activation increased (Figure 3A). Although PSK alone had no significant effect on NF- $\kappa$ B activation, PSK suppressed the NF- $\kappa$ B activation induced by the TXT treatment (Figure 3B).

**Effect of TXT and PSK on growth, apoptosis and migration ability of the MK-1 cells.** PSK below 100  $\mu$ g/ml or TXT below 1 nM did not significantly affect the proliferation of the MK-1 cells, however, a combination of PSK (100  $\mu$ g/ml) and TXT (1 nM) significantly suppressed their proliferation (Figure 4A). When TXT was used at 1 nM, PSK greater than 50  $\mu$ g/ml enhanced the growth inhibition induced by TXT (Figure 4B). Significant apoptosis in the MK-1 cells was induced by TXT at 10 nM but not by PSK below 100  $\mu$ g/ml. As expected, PSK enhanced the apoptosis induced by TXT, even at a concentration of 10  $\mu$ g/ml (Figure 5). Treatment with 1 nM TXT significantly enhanced the invasive activity of the MK-1 cells (Figure 6B) compared with that of the control (Figure 6A). Importantly, PSK (50  $\mu$ g/ml) reduced the enhanced invasion induced by the TXT (Figure 6C).

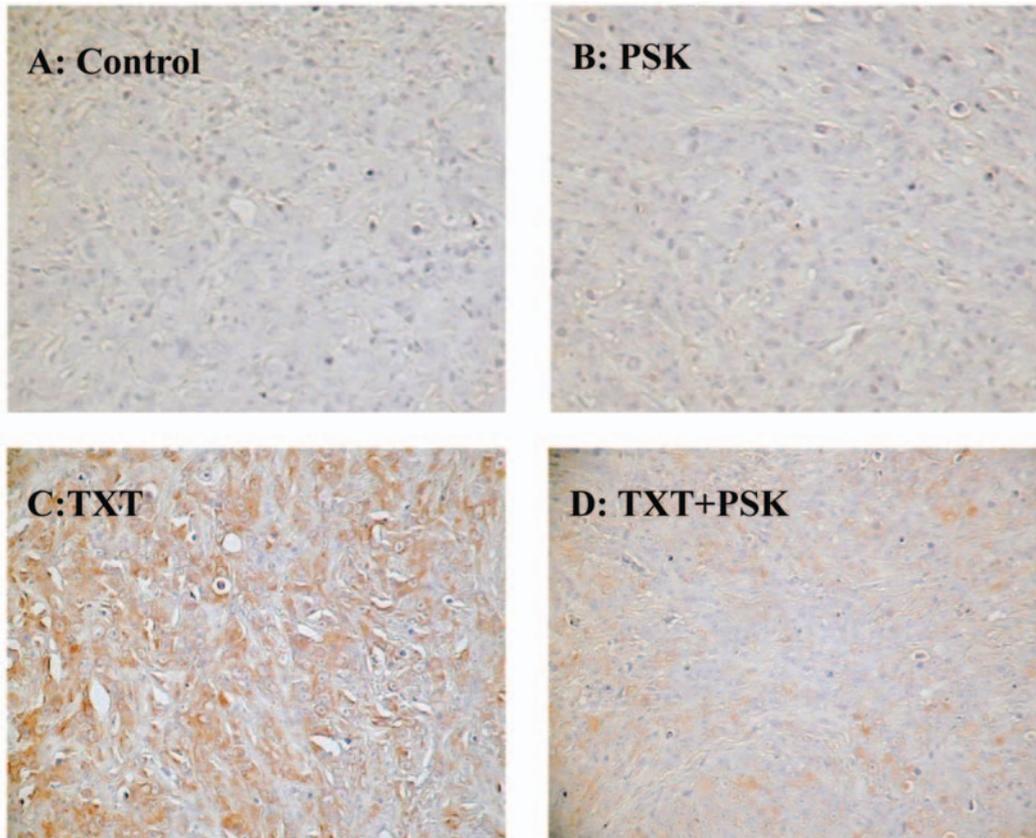


Figure 2. Effect of TXT and PSK on NF- $\kappa$ B activation in tumors. NF- $\kappa$ B activation examined immunohistochemically using an anti-NF- $\kappa$ B p65 antibody in tumors from control mice (A) and from mice injected with PSK alone (B), TXT (C) or TXT and PSK (D).

**Effect of PSK and TXT on MMP-9 mRNA expression.** PSK (100  $\mu$ g/ml) was added to MK-1 cells 1 h before treatment with TXT (1 nM) for 8 h. TXT enhanced MMP-9 mRNA expression and this enhancement was suppressed by PSK (Figure 7).

## Discussion

The present study showed for the first time that PSK can enhance the antitumor efficacy of TXT in a gastric cancer xenograft model. TXT induced NF- $\kappa$ B activation in the growing tumors and PSK reduced this TXT-induced NF- $\kappa$ B activation.

Our previous studies indicated that PSK could suppress NF- $\kappa$ B activation induced by TXT in cancer cells, including gastric cancer and pancreatic cancer cells (12, 13). Recent studies have shown that *MDR1*, one of the genes involved in MDR, is one of the downstream genes of NF- $\kappa$ B (28, 29). Thus, PSK might enhance the antitumor effect of TXT by inhibiting MDR through inhibition of the NF- $\kappa$ B activation induced by TXT. As expected, PSK enhanced the TXT-mediated antitumor effect in a mouse model of human

gastric cancer (Figure 1). In addition, TXT induced NF- $\kappa$ B activation in both the *in vivo* growing tumors and *in vitro* growing MK-1 cells, and PSK reduced this TXT-induced NF- $\kappa$ B activation (Figures 2 and 3). In addition, PSK enhanced the TXT-mediated growth suppression and apoptosis of MK-1 cells growing *in vitro* (Figures 4 and 5). When an NF- $\kappa$ B inhibitor, PDTC (30), instead of PSK was combined with TXT, a similar enhancement of apoptosis was found (data not shown), suggesting a contribution of NF- $\kappa$ B inhibition by PSK to the enhanced TXT-induced apoptosis. In fact, we have shown previously that PSK enhanced TXT-induced apoptosis through suppression of NF- $\kappa$ B activation in human pancreatic cancer cells (12). In that report, a possible contribution of cellular inhibitor of apoptosis protein (cIAP-1), which is transcriptionally regulated by NF- $\kappa$ B and functions as an anti-apoptotic molecule (31), to the enhanced apoptosis was indicated (12). However, no data were obtained concerning the expression of cIAP-1 in the *in vivo* growing tumors.

NF- $\kappa$ B activation has been linked to the invasive ability of cancer cells, including gastric carcinoma cells, because of a close relationship between NF- $\kappa$ B activation and increased

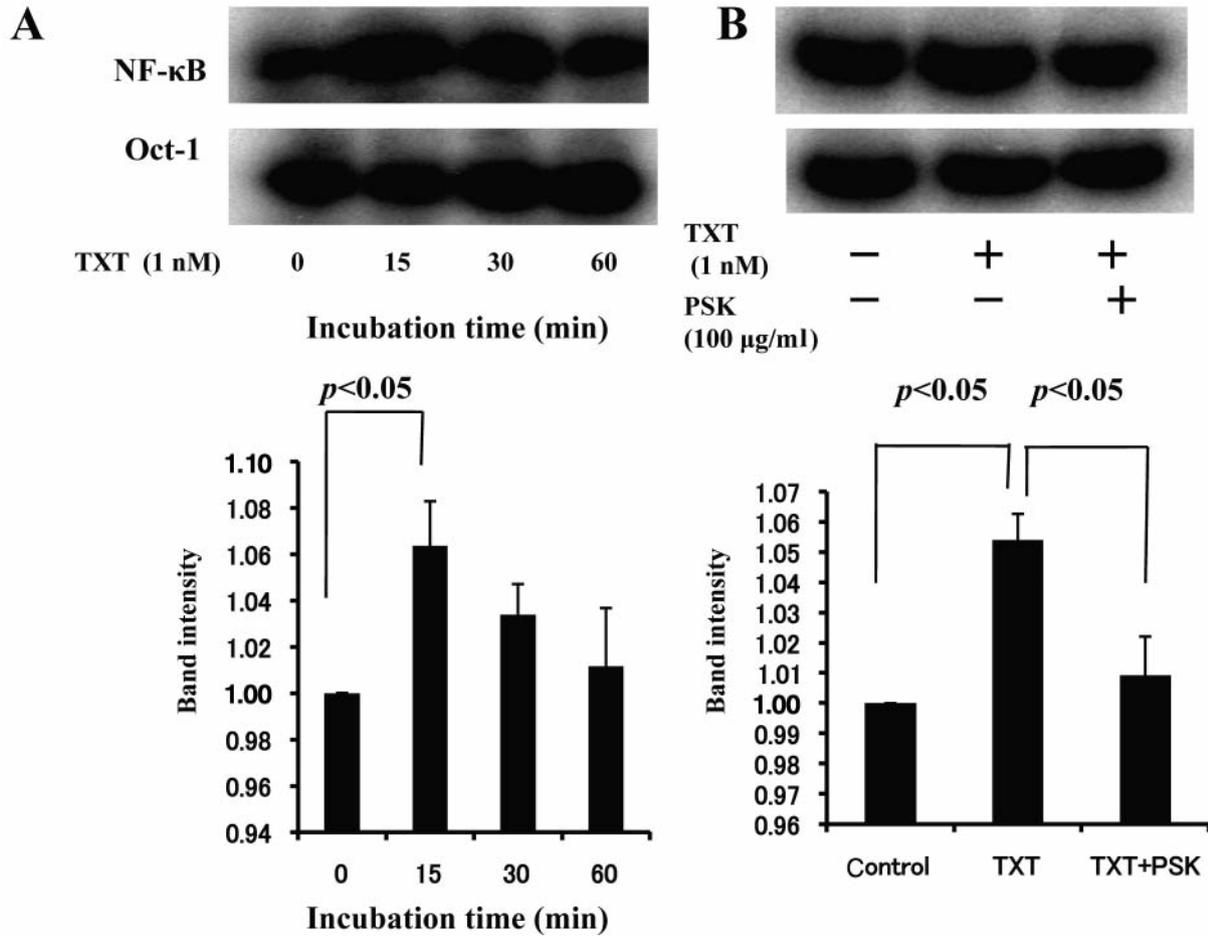


Figure 3. Effect of TXT and PSK on NF-κB activation in the MK-1 cells. A, EMSA evaluation of NF-κB activation by TXT. B, TXT-induced NF-κB activation with or without PSK. Band intensities of nuclear factor-κB were quantified with Image J version 1.39 software (NIH Division of Computer Research and Technology) and the values represent the mean ± SD from three different experiments. The control band intensity was designated as 1.0.

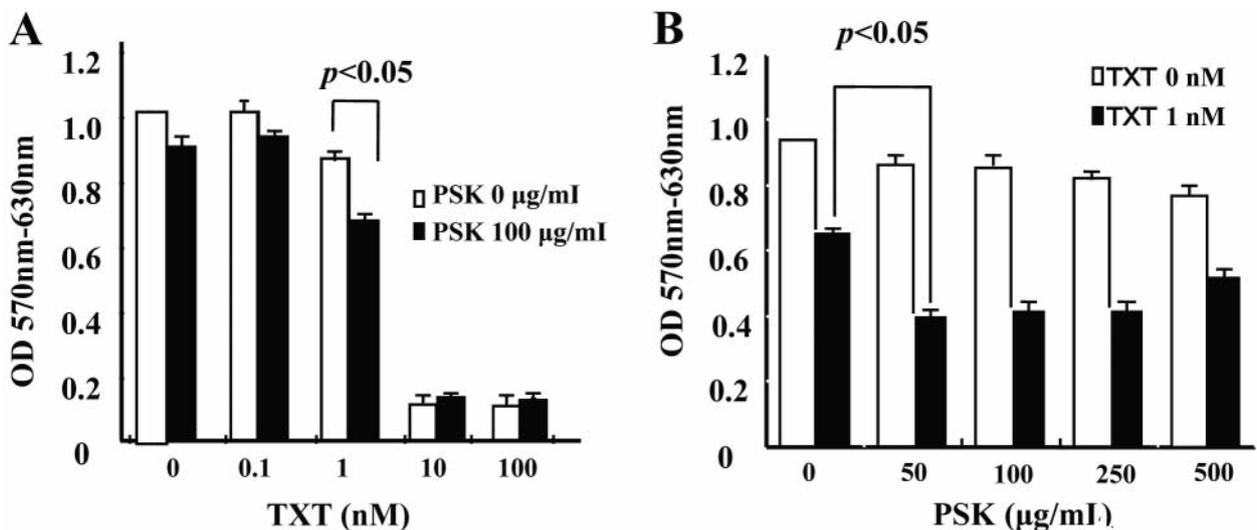


Figure 4. PSK enhances the growth suppression of MK-1 cells induced by TXT. MK-1 cells were incubated for 48 h under different culture conditions. Cell growth was estimated by the MTT assay.

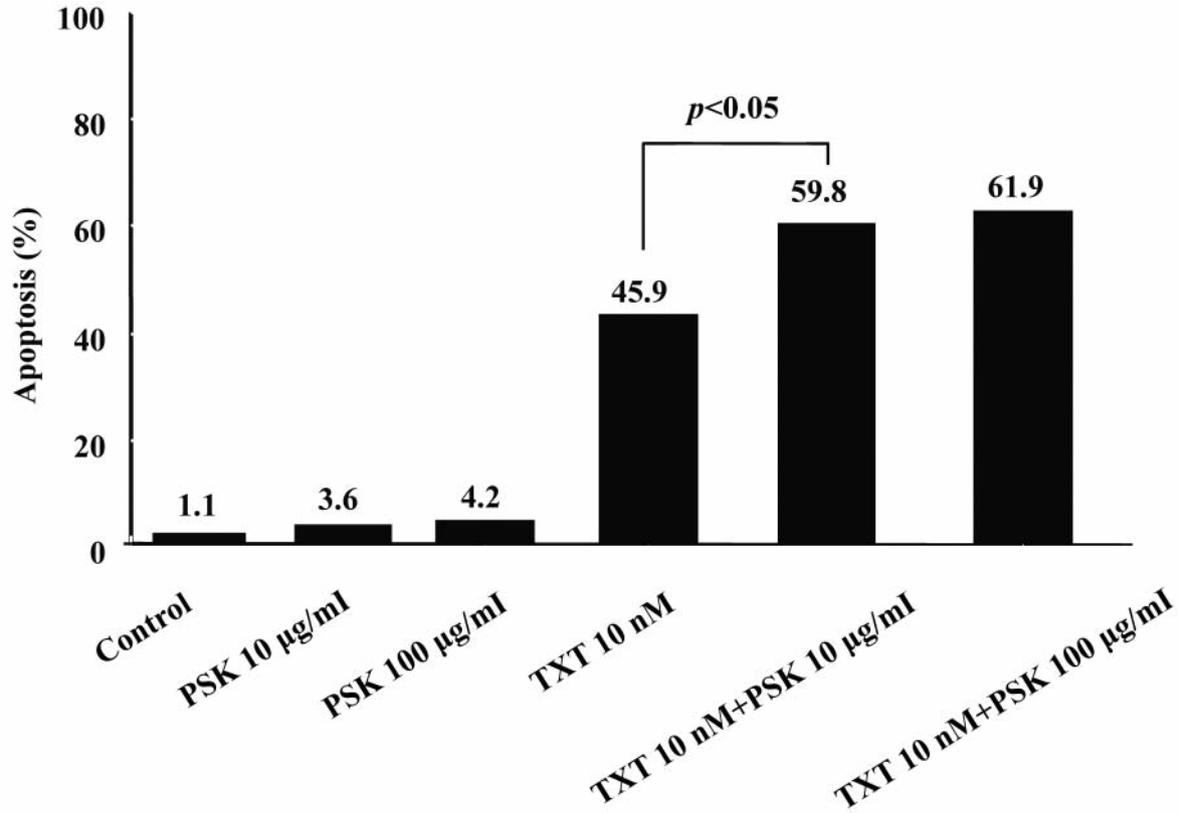


Figure 5. Effect of TXT and PSK on apoptosis of MK-1 cells. Apoptotic MK-1 cells were determined by Hoechst 33342 staining after 24 h incubation.

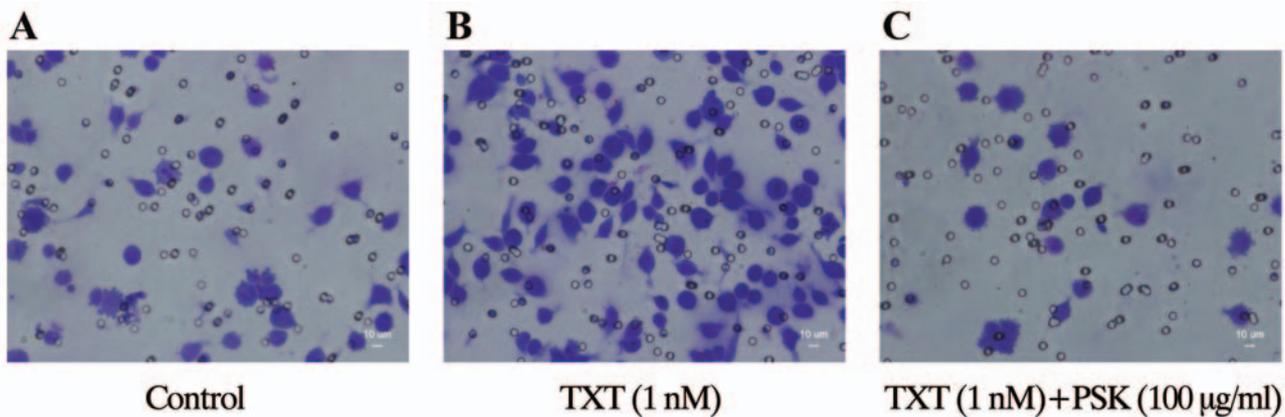


Figure 6. Effect of PSK on the enhanced MK-1 cell invasion induced by TXT. MK-1 cells that migrated from the upper to the lower side of the matrigel-coated filter.  $\times 80$  magnification.

expression of invasion-related molecules, such as the MMPs, in cancer cells (32, 33). TXT enhanced the invasive ability of the MK-1 cells in the present study (Figure 5). We have previously reported that IL-1 $\beta$  enhanced NF- $\kappa$ B activation, MMP-9 expression and the invasion of gastric cancer cells

(32). Our previous study also showed that PSK inhibited MK-1 cells by the down-regulation of transforming growth factor- $\beta$  and MMPs such as MMP-2 and MMP-9 (34). Taken together, it seems likely that TXT induces the expression of MMPs through NF- $\kappa$ B activation and that PSK reduces the

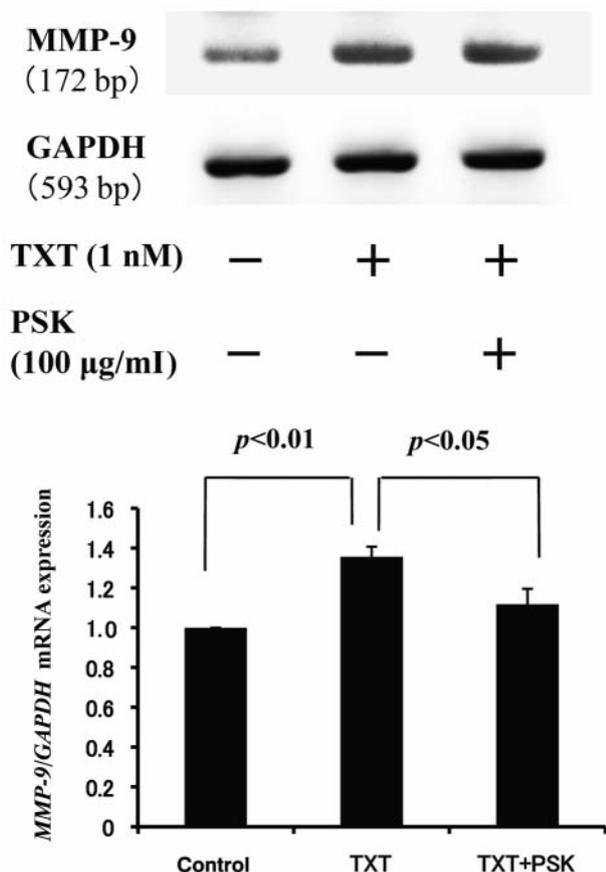


Figure 7. Effect of TXT and PSK on MMP-9 mRNA expression. MMP-9 mRNA expression was determined by semiquantitative reverse transcription-PCR. Band intensities were quantified with Image J. The control band intensity was designated as 1.0.

enhanced TXT-induced MMP expression, as was shown at the mRNA level in the MK-1 cells (Figure 7).

In conclusion, the data presented here may support combination therapy of TXT with PSK, which is planned as a phase III study in Japan, for patients with gastric cancer.

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Received May 28, 2008

Revised August 20, 2008

Accepted November 10, 2008