Molecular Basis for the Induction of an Angiogenesis Inhibitor, Thrombospondin-1, by 5-Fluorouracil

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Abstract

5-Fluorouracil (5-FU) is one of the most commonly used anticancer drugs in chemotherapy against various solid tumors. 5-FU dose-dependently increased the expression levels of intrinsic antiangiogenic factor thrombospondin-1 (TSP-1) in human colon carcinoma KM12C cells and human breast cancer MCF7 cells. We investigated the molecular basis for the induction of TSP-1 by 5-FU in KM12C cells. Promoter assays showed that the region with the Egr-1 binding site is critical for the induction of TSP-1 promoter activity by 5-FU. The binding of Egr-1 to the TSP-1 promoter was increased in KM12C cells treated with 5-FU. Immunofluorescence staining revealed that 5-FU significantly increased the level of Egr-1 in the nuclei of KM12C cells. The suppression of Egr-1 expression by small interfering RNA decreased the expression level of TSP-1. Furthermore, 5-FU induced the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and heat shock protein 27 (HSP27). Blockade of the p38 MAPK pathway by SB203580 remarkably inhibited the phosphorylation of HSP27 induced by 5-FU and decreased the induction of Egr-1 and TSP-1 by 5-FU in KM12C cells. These findings suggest that the p38 MAPK pathway plays a crucial role in the induction of Egr-1 by 5-FU and that induced Egr-1 augments TSP-1 promoter activity, with the subsequent production of TSP-1 mRNA and protein.

Introduction

5-Fluorouracil (5-FU) is a commonly used anticancer drug in chemotherapy against various solid tumors (1). Recent clinical studies have shown that UFT (a prodrug of 5-FU, Tegafur, combined with uracil in a 1:4 molar ratio) is an active oral chemotherapeutic agent in postoperative adjuvant settings for completely resected early-stage lung, gastric, colorectal, and breast cancer that does not exhibit any remarkable toxicity (2). UFT can achieve a higher maximum plasma 5-FU level for a longer period by inhibiting 5-FU degradation, thereby enhancing its antitumor effect (3). Angiogenesis is an important therapeutic target for a variety of malignant tumors. UFT-containing long-term chemotherapy significantly improved patient survival (4). The antiangiogenic effect of UFT might contribute, at least in part, to its clinical efficacy. Recently, we examined the antitumor and antiangiogenesis activities of the 5-FU–based drug, S-1 (1 mol/L teagafur, 0.4 mol/L 5-chloro-2,4-dihydroxypyridine, and 1mol/L potassium oxonate), at a sub–maximum tolerated dose (sub-MTD) on human colorectal cancer xenografts. The up-regulation of thrombospondin-1 (TSP-1), as well as down-regulation of microvessel formation, has been shown (5). However, the molecular basis for the suppression of angiogenesis by 5-FU has not been fully elucidated (6).

TSP-1 has been shown to inhibit angiogenesis by inhibiting endothelial cell migration, inducing endothelial cell apoptosis, directly interacting with vascular endothelial growth factor (VEGF), and inhibiting matrix metalloproteinase-9 activation (7). Furthermore, 5-FU can inhibit angiogenesis by decreasing the level of circulating endothelial cell progenitors (8). However, the molecular basis for TSP-1 induction by 5-FU and other anticancer agents is unknown (9).

In the present study, we found that 5-FU induced TSP-1 in human colon carcinoma KM12C cells. A transcription factor, Egr-1, was also induced by 5-FU and bound to the promoter of TSP-1, enhancing its transcription and the subsequent production of TSP-1 protein. Moreover, we present the evidence that p38 mitogen-activated protein kinase (MAPK) plays an important role in 5-FU–induced Egr-1 transactivation.

Materials and Methods

Reagents and antibodies. 5-FU was provided by Taiho Pharmaceutical Co., Ltd. SB203580 was obtained from Calbiochem. An antibody against Egr-1 was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibodies against a-tubulin and TSP-1 were purchased from Oncogene and NeoMarkers, respectively. Anti–heat shock protein 27 (HSP27) G3 monoclonal and anti–phosphorylated HSP27 antibodies were obtained from Cell Signaling Technology.

Cell lines and cell cultures. KM12C human colon cancer cells were provided by Dr. Kiyoshi Morikawa (Iwamizawa Worker's Compensation Hospital), LOVO human colon cancer cells were purchased from Dainippon Seiyaku Co., Ltd., and MCF7 breast cancer cells were obtained from National Cancer Institute. The cells were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum.

RNA isolation and cDNA synthesis. KM12C cells were treated with various concentrations of 5-FU for various periods, as described. The total RNA from the cultured cells were isolated using TRIzol (Invitrogen),
The expression levels of TSP-1 mRNA in KM12C cells were measured using real-time PCR. The expression of TSP-1 at 1 and 2 d in the absence or presence of 5-FU (0.5 and 1 mol/L) for 5 d and then harvested, and the effect of the siRNA on the expression of TSP-1 and Egr-1 was assessed using real-time PCR, RT-PCR, and immunoblotting, as described above.

**TSP-1 promoter-reporter constructs.** Three deletion mutants of pGL3-TSP-1, namely (−1,210/+750), (−667/+750), and (−71/+750), were prepared as described previously (10).

The pGL3-TSP-1 (−2,033/+750) plasmid was constructed as follows. The TSP-1 promoter, ranging from −2,033 to +750, was amplified using PCR with KOD plus polymerase (Toyobo) from the genomic DNA of KM12C cells using a sense primer (5'-CGCGTCTAGCCTGACTCGAGCATCCTGACTTTCAAATCCCTAC-CAATCCCCTACG-3') and an antisense primer (5'-CGCGTCTAGCATCGCTTGGTACTGCTTAGCGAAGACGCGAAGCG-3'). These primers contained an Nhel or an XhoI site at the 5' end, respectively.

The pGL3-TSP-1 (−2,033/+750 ΔEgr-1) plasmid was derived from pGL3-TSP-1 (−2,033/+750), deleting the Styl Not1 region, including the Egr-1 binding site.

**Transient transfection and dual luciferase reporter assay.** KM12C cells were plated at a density of 1 × 10^5 per well in 24-well plates and pretreated with various concentrations of 5-FU for 3 d before transfection. Transfection and dual-luciferase assay were performed as described previously (11).

**Chromatin immunoprecipitation assay.** Cells treated with 5-FU, as described above, were fixed with 1% formaldehyde for 10 min at 37°C to cross-link protein to DNA. A chromatin immunoprecipitation (ChIP) assay was carried out using a ChIP assay kit (Upstate Biotechnology), according to the manufacturer's instructions. The soluble DNA fraction was mixed with an anti-Egr-1 antibody or nonimmunized mouse IgG (Santa Cruz Biotechnology), and the precipitated DNA was amplified with primers for the TSP-1 promoter [5'-AACAGATTGGCTCTCCTTGGTGT-3' (sense) and 5'-CTTCCAGTAGAAAGTA -3' (antisense)].

**Confocal fluorescence microscopy.** KM12C cells (7.5 × 10^5) were cultured in the medium with or without 2 μmol/L 5-FU for 5 d on coverslips, fixed with 3% formaldehyde in PBS for 10 min at room temperature, and permeabilized with 100% methanol for 10 min. Cells were incubated with an antibody against Egr-1 at 4°C overnight. After washing thrice in PBS, the cells were incubated with 200-fold diluted Alexa Fluor 546-labeled antirabbit IgG (Invitrogen). Nuclei were stained by incubating cells with 6 μmol/L 4',6-diamidino-2-phenylindole (DAPI). The cells were observed using confocal fluorescence microscopy (FV500, Olympus Corporation).

**Statistical analysis.** Statistical comparisons were performed using the Student's t test. Quantitative data were expressed as the means ± SD. P < 0.05 was considered significant.

**Results**

Expression of TSP-1 protein induced by 5-FU in human colon cancer cell lines. Our recent results have shown that the proteins in the whole cell lysate (200 μg) were separated using SDS-PAGE. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane and reacted with primary antibodies against TSP-1, Egr-1, phosphorylated HSP27, α-tubulin, and HSP27. After incubation, membranes were washed and incubated with antiserum or antirabbit secondary antibodies (GE Health Science). The membranes were developed using the enhanced chemiluminescence detection system (Amersham Biosciences).

**Inhibition of Egr-1 expression by Egr-1 small interfering RNA.** Egr-1–specific small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology. Transfections (40 μmol/L Egr-1 siRNA) were accomplished using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. As a control, the cells were treated with an equal amount of GFP (eGFP) siRNA (Ambion). After transfection, the cells were exposed to 5-FU (1 and 2 μmol/L) for 5 d and then harvested, and the effect of the siRNA on the expression of TSP-1 and Egr-1 was assessed using real-time PCR, RT-PCR, and immunoblotting, as described above.

**Figure 1.** TSP-1 and TSP-1 mRNA expression induced by 5-FU in human colon cancer cell lines. A, effect of 5-FU on TSP-1 protein levels in human colon cancer cell lines. Cells were treated with 5-FU at 0.5 and 1 μmol/L for 4 d. An immunoblot analysis was performed using an antibody against TSP-1. α-Tubulin was used as a loading control. B, quantification of relative TSP-1 protein levels. The staining intensities of the bands for TSP-1 and α-tubulin were quantified using NIH image. Protein levels of TSP-1 were normalized to α-tubulin protein levels. Expression levels of TSP-1 are shown relative to that in the untreated cells. Columns, average of three independent experiments; bars, SD. *, P < 0.05; **, P < 0.01, significantly different from untreated cells. C, effect of 5-FU on TSP-1 mRNA levels in KM12C cells. Cells were treated with 5-FU at 1 and 2 μmol/L for 1, 3, and 5 d. The relative expression levels of TSP-1 mRNA in KM12C cells were measured using real-time PCR. The expression of the GAPDH gene was used to normalize the values of TSP-1. Data are expressed relative to the TSP-1 mRNA 5-FU–untreated cells at day 1 (considered 1). Columns, average of three independent experiments; bars, SD. *, P < 0.05; **, P < 0.01, significantly different from untreated cells on the same day.
5-FU–based drug, S-1, at sub-MTD concentration has antiangiogenic function through up-regulation of TSP-1 in colorectal cancer xenografts (5). To investigate possible mechanisms underlying the specificity of this effect, human colon cancer cells were treated with 5-FU at concentrations near or below IC50 for 4 days (Supplementary Fig. S1). The expression of TSP-1 in two human colon cancer cell lines was then determined by immunoblot analysis using an antibody against TSP-1. When KM12C and LOVO cells were treated with 5-FU at 1 μmol/L, the expression levels of TSP-1 were 3-fold and 2-fold increased, compared with counterpart untreated cells, respectively (Fig. 1 A and B). Meanwhile, treatment of the cells with 10 μmol/L 5-FU for 1 day also increased the expression of TSP-1 protein in KM12C cells (data not shown).

In this study, we focused on the molecular basis for the induction of TSP-1 by low-dose 5-FU. Because the TSP-1 protein level in KM12C cells treated with 5-FU was considerably higher than that in 5-FU–treated LOVO cells, we used KM12C cells for further study.

Effect of 5-FU on the expression of the TSP-1 gene in KM12C cells. The levels of TSP-1 mRNA in KM12C cells incubated in the absence or presence of 5-FU were determined using real-time RT-PCR. Treatment of the KM12C cells with 5-FU at 1 and 2 μmol/L for 1, 3, and 5 days increased the TSP-1 mRNA levels in a dose-dependent and time-dependent manner compared with those in untreated cells (Fig. 1C). Furthermore, the expression level of TSP-1 mRNA in human umbilical vein endothelial cells treated with 5-FU was ~ 2-fold higher than in the untreated cells (data not shown). These data suggested that 5-FU enhanced TSP-1 protein expression in both cancer and endothelial cells by activating transcription of TSP-1 gene.

Identification of the transcriptional regulatory element necessary for transcriptional activation of the TSP-1 gene by 5-FU. To investigate which transcriptional regulatory elements in the TSP-1 promoter contribute to the transcriptional activation of the TSP-1 gene by 5-FU, we made wild-type and various deletion constructs of the TSP-1 promoter (Fig. 2A). The 5-flanking region up to −2,033 from the transcription initiation site contained several putative binding sites for known transcription factors, including an Egr-1 site and Sp-1 sites (GC boxes; Fig. 2A). The longest construct (−2,033/+750) showed the highest promoter activity among the constructs in the cells treated with 5-FU, and the activity was considerably decreased when the constructs lacked the region including both the Egr-1 and Sp-1 binding sites (Fig. 2B).

These findings suggested that Egr-1 or Sp-1 transcription factors might enhance the expression of the TSP-1 gene.

TSP-1 promoter and its in vivo Egr-1 recruitment. DNA-damaging agents can up-regulate the expression of the tumor suppressor gene Egr-1 in both normal and cancer cells (12), whereas Sp-1 plays a role in the EGF-induced activation of the TSP-1 gene (10). We thus focused our study on Egr-1. To confirm the recruitment of Egr-1 to the TSP-1 promoter in vivo, a ChIP assay was performed (Fig. 2C). The Egr-1 recruitment was dose-dependently and time-dependently enhanced by 5-FU in KM12C cells. This enhancement was hardly detected when nonimmune IgG was used (Fig. 2C, bottom). These results indicated the enhanced binding of Egr-1 to the TSP-1 promoter in cells treated with 5-FU.

Effect of Egr-1 knockdown on the induction of TSP-1 by 5-FU. To confirm that Egr-1 is involved in the enhanced expression of TSP-1 by 5-FU, Egr-1 siRNAs were used to knockdown the expression of Egr-1. The induction of Egr-1 mRNA and protein expression by 5-FU was considerably suppressed by Egr-1 siRNA, but not by GFP siRNA. Egr-1 knockdown resulted in the decreased expression of TSP-1 mRNA and protein (Fig. 4A–D). These results show that Egr-1 is required for the 5-FU–mediated induction of TSP-1.

To examine whether Egr-1 is required for the 5-FU–mediated induction of TSP-1 in other tumor cells, we determined the expression levels of Egr-1 and TSP-1 in MCF7 cells. When MCF7 cells were treated with 1 and 2 μmol/L 5-FU at concentrations near IC50 (Supplementary Fig. S2A), the expressions of Egr-1 and

Figure 2. Effect of 5-FU on TSP-1 promoter activity. A, schematic representation of a series of TSP-1 promoter deletion constructs. The Egr-1–binding site is indicated by the closed square. B, KM12C cells were pretreated for 3 d with 5-FU, and these constructs were transiently transfected to the cells using Lipofectamine. The cells were further incubated with or without 2 μmol/L 5-FU for 48 h and harvested, and their luciferase activities were determined. The luciferase activities were corrected for differences in transfection efficiency among wells, as estimated using the Renilla luciferase activities. Columns, representative of triplicate independent experiments; bars, SD. * P < 0.05, significantly different from untreated cells transfected with the same construct. C, binding of Egr-1 to TSP-1 promoter in KM12C cells. KM12C cells were treated with 5-FU at 1 and 2 μmol/L for 1, 3, and 5 d. The cells were fixed with formaldehyde to form a DNA-protein complex and were subjected to a ChIP assay, as described in the Materials and Methods. PCR for the core promoter region of the TSP-1 gene was performed using DNA extracted from the DNA-protein complex immunoprecipitated using an anti–Egr-1 antibody or nonimmune IgG.
TSP-1 were also increased compared with those of untreated cells (Supplementary Fig. S2B–D). TSP-1 protein levels in the 5-FU–treated MCF7 cells were suppressed when Egr-1 was down-regulated by Egr-1 siRNA (data not shown). These results suggest that our findings are not limited to KM12C cells.

Effect of 5-FU on the activation of the p38 MAPK pathway.
Several studies have indicated the activation of one or more members of the MAPK family of intracellular signaling kinases by cytotoxic agents (13). Among them, p38 often transmits the signal generated by diverse stimuli (14). The p38 MAPK inhibitor SB203580 attenuated the TSP-1 up-regulation induced by trastuzumab and transforming growth factor-β1 (TGF-β1; refs. 10, 15). We thus focused our study on p38 MAPK. To examine whether 5-FU is involved in the activation of the MAPK pathways, the effect of 5-FU on the phosphorylation of proteins involved in p38 MAPK pathways was studied. The treatment of KM12C cells with 1 and 2 μmol/L 5-FU for 5 days increased the phosphorylation of p38, whereas the expression level of p38 remained unchanged (Fig. 5A). This indicated that the p38 MAPK pathway is activated by 5-FU.

Effect of p38 MAPK inhibitor on 5-FU–induced Egr-1 and TSP-1 expression.
To confirm that the activation of p38 MAPK contributes to 5-FU–induced Egr-1 and TSP-1 expression, we examined the effect of a p38 MAPK inhibitor SB203580 on the expression of Egr-1 and TSP-1. As shown in Fig. 5, the p38 MAPK inhibitor SB203580 remarkably suppressed the expression of Egr-1, TSP-1 mRNA, and Egr-1 (Fig. 5B–D). The p38 MAPK signaling pathway might play an important role in the 5-FU–induced expression of Egr-1 and TSP-1 in KM12C cells. In accordance with these findings, SB203580 attenuated the 5-FU–induced phosphorylation of HSP27, one of the downstream molecules of p38 MAPK. However, it had no effect on 5-FU–induced HSP27 expression (Fig. 5D).

Discussion
Previous studies indicated that TSP-1 induced by low-dose cyclophosphamide is implicated in the suppression of tumor growth (16). Furthermore, our recent study showed that 5-FU-based drugs have antitumor function partially through up-regulation of TSP-1 in colorectal cancer xenografts (5). In accordance with these results, we also found that 5-FU enhanced the expression of TSP-1 in human colon cancer (KM12C and LOVO cells) and breast cancer MCF7 cells (Fig. 1 and Supplementary Fig. S2B and D). The expression level of TSP-1 mRNA in human umbilical vascular endothelial cell was also increased by 5-FU (data not shown). Various extracellular stimuli and compounds altered TSP-1 gene expression (17). It is generally accepted that TSP-1 expression levels are tightly regulated at the transcriptional level. Donoviel and colleagues (18) identified the TSP-1 promoter region and found that the 5'-flanking region between -234 and +750 was important for the basal transcriptional activity (19). The promoter region of mouse TSP-1 contains one Egr-1 binding site, and TSP-1 transcription is enhanced by
In human hepatic HuH-7 cells, the TSP-1 promoter region between −267 and −71 contained two GC boxes to which Sp-1 bound. These boxes were found to be responsible for the promoter activity enhanced by EGF (10). Consistent with these studies, we also showed that TSP-1 promoter region (−267/−71) is needed for the augmentation TSP-1 promoter activity by 5-FU, and the deletion of the region in which the Egr-1 and Sp-1 binding sites reside almost completely blocked the TSP-1 expression.

**Figure 4.** Effect of Egr-1 knockdown on the induction of TSP-1 in KM12C cells treated with 5-FU. KM12C cells were transfected with 40 nmol/L Egr-1 siRNA for 6 h and then subjected to 5-FU at 2 μmol/L for 5 d. A, semiquantitative RT-PCRs performed with primers specific for the indicated genes. B, the levels of Egr-1 (left) and TSP-1 (right) mRNAs were measured using real-time RT-PCR. The GAPDH gene was used to normalize the values of TSP-1 and Egr-1 mRNAs. Data are expressed relative to the Egr-1 or TSP-1 mRNA level in GFP siRNA-transfected and 5-FU–untreated cells. Columns, an average of three independent experiments; bars, SD. *, P < 0.05; **, P < 0.01, significantly different from GFP siRNA-transfected cells treated with the same concentrations of 5-FU. C, the expression level of Egr-1 and TSP-1 in 5-FU–treated KM12C cells transfected with GFP or Egr-1 siRNA was analyzed. The cell lysates were subjected to SDS-PAGE, and the expressions of Egr-1 and TSP-1 were detected using immunoblotting with the antibodies indicated in Materials and Methods. D, Egr-1 (left) and TSP-1 (right) protein levels were determined as described in Fig. 1B. Data are expressed relative to the levels of Egr-1 or TSP-1 in GFP siRNA-transfected and 5-FU–untreated cells. Columns, an average of three independent experiments; bars, SD. *, P < 0.05; **, P < 0.01, significantly different from GFP siRNA-transfected cells untreated with 5-FU.

**Figure 5.** Activation of p38 MAPK pathway by 5-FU and the effect of the activated p38 MAPK pathway on the 5-FU–induced expression of TSP-1 mRNA levels and Egr-1 expression in KM12C cells. A, KM12C cells were exposed to 1 or 2 μmol/L of 5-FU for 5 d, and an immunoblot analysis was performed using an antibody against phosphorylated p38 kinase. The blot was reprobed with an antibody against p38. KM12C cells were treated as described above, and 5-FU–induced Egr-1 and TSP-1 mRNA levels were measured using semiquantitative RT-PCR and real-time RT-PCR. B, semiquantitative RT-PCRs performed with primers specific for the indicated genes. C, relative expression levels of Egr-1 (left) and TSP-1 (right) mRNAs in KM12C cells were measured using real-time RT-PCR. The expression of the GAPDH gene was used to normalize the values of Egr-1 and TSP-1. Data are expressed relative to the Egr-1 or TSP-1 mRNA in the untreated cells. Columns, an average of three independent experiments; bars, SD. *, P < 0.05; **, P < 0.01, significantly different compared with the cells treated with 2 μmol/L 5-FU alone. D, KM12C cells were exposed to 2 μmol/L 5-FU with or without MAPK inhibitor for 5 d, and an immunoblot analysis was performed using the indicated antibodies. α-Tubulin was used as an internal control.
results showed that 5-FU activated p38 MAPK (Fig. 5). c-Jun amino-terminal kinase and p38 MAPK pathways have been implicated in the response to chemotherapeutic drugs (28). Egr-1 binds to the promoter of TSP-1 and enhances the transcription of the TSP-1 gene and subsequently the expression of Egr-1.

Egr-1 is a Cys2-His2-type zinc-finger transcription factor and binds to GC-rich, cis-acting promoter elements, controlling the expression of a wide variety of pathogenesis-relevant genes, encoding growth factors, cytokines, receptors, adhesion molecules, and proteases, many of which are involved in angiogenesis, tumorigenesis (21), the response to ischemia (22), and the progress of several vascular diseases (23). A number of reports also indicate that Egr-1 acts as a tumor suppressor gene. Egr-1 is down-regulated in several types of neoplasia, as well as an array of tumor cell lines. Egr-1 is induced very early in the apoptotic process, where it mediates the activation of downstream regulators, such as p38 (24). Egr-1 also activates phosphatase and tensin homologue tumor suppressor gene during UV irradiation (25), suppressing the growth of transformed cells in both soft agar and athymic nude mice (26). Sustained Egr-1 expression may cause the induction of multiple pathways of antiangiogenesis, growth arrest, and apoptosis induction in proliferating cells leading to preferential inhibition of angiogenesis and tumor growth. Egr-1 possesses a strong inhibitory effect on the angiogenic activity of VEGF in vivo (27). Our results showed that Egr-1 induced by 5-FU was needed for the increased expression of TSP-1 in KM12C cells treated with 5-FU (Figs. 3 and 4).

MAPK pathways have been implicated in the response to chemotherapeutic drugs (28). c-Jun amino-terminal kinase and p38 kinase are important for controlling cell growth and apoptosis in response to chemical stress, radiation, and growth factors (29). Our results showed that 5-FU activated p38 MAPK (Fig. 5A). Activated p38 MAPK phosphorylates MAPKAP kinase 2, which in turn phosphorylates HSP27 (30). SB203580 attenuated the 5-FU-enhanced phosphorylation of HSP27, indicating that SB203580 effectively inhibited the activation of p38 pathway in 5-FU-treated KM12C cells. SB203580 suppressed the expression of both Egr-1 and TSP-1 mRNAs, suggesting that the activation of the p38 MAPK pathway by 5-FU is responsible for the induction of Egr-1 and TSP-1 (Fig. 5B–D). Trastuzumab also stimulated sustained p38 activation, and SB203580 attenuated the TSP-1 up-regulation induced by trastuzumab (15). SB203580 partially inhibited TGF-β1–induced TSP-1 expression (10). These findings suggest that the activation of p38 MAPK plays an important role in the induction of TSP-1 by some anticancer agents and growth factors. Further study is needed to determine the detailed mechanisms underlying the regulation of the p38 MAPK pathway by 5-FU.

Several transcription factors are regulated by p38 MAPK, and this kinase is involved in the control of the expression of various genes. In vitro studies show that the transcription factor ATF2 is phosphorylated and activated by p38 MAPK. In addition, p38 MAPK activates the Elk-1, CHOP, MEF2C, and SAP-1 transcription factors (31). Our finding that 5-FU activated p38 MAPK and then increased the expression of Egr-1 may be useful for elucidating the molecular basis for the chemopreventive and antitumor effects of 5-FU and its prodrugs.

Egr-1 binds to the promoter of TSP-1 in cells treated with 5-FU was also observed (Fig. 2C).

Egr-1 activates phosphatase and tensin homologue tumor suppressor gene and subsequently the expression of Egr-1.

A schematic representation of a proposed molecular basis for the up-regulation of TSP-1 by 5-FU in KM12C cells is shown in Fig. 6. Our findings show that 5-FU activated p38 MAPK and then up-regulated Egr-1 expression, resulting in the expression of an endogeneous antiangiogenic factor, TSP-1. Recently, we have found that the expression of VEGF mRNA was suppressed by 5-FU using Genechip analysis (11). VEGF is produced in varying quantities in tumors and seems to be an important modulator of TSP function. Relative ratios of TSPs to VEGF might determine whether vessels regress or proliferate (17). Further study is needed to elucidate whether TSP-1 induced by 5-FU is involved in the antitumor effect of 5-FU. A better understanding about the mechanisms of the antiangiogenic and the antitumor effect of 5-FU might provide new approaches for the treatment of colon and breast cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 6. Schematic pathway for 5-FU–induced TSP-1 expression in KM12C human colon cancer cells. In this model, 5-FU causes the activation of the p38 MAP kinase pathway, leading to the increased expression of Egr-1. Egr-1 binds to the promoter of TSP-1 and enhances the transcription of the TSP-1 gene and subsequently the expression of Egr-1.


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