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The PI3K-Akt Pathway in SN-38-Induced Apoptosis in Human Gastric Cancer Cell Lines

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SN-38, an active metabolite of a topoisomerase I inhibitor, CPT-11, exhibits a cytotoxic effect by inducing apoptosis in cancer cells. Phosphatidylinositol-3-OH kinase (PI3K)-Akt signaling is known to protect a variety of cells from apoptosis. The relationship between resistance to SN-38-induced apoptosis and the PI3K-Akt pathway in human gastric cancer cells is unknown. Here, we did an investigation using two gastric cancer cell lines, MKN1 and MKN45. Cell viability was determined by sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay. Apoptosis was confirmed by fluorescence microscopy using Hoechst 33342 staining. Expression levels of phospho-Akt (pAkt) were determined by Western blotting. After being treated with SN-38, the populations of sub-G1 cells were induced by flow cytometry in 36.8% of MKN45 cells more frequently than in 13.5% of MKN1 cells. SN-38 inhibited the expression of pAkt dose-dependently in MKN45 cells, but not in MKN1 cells. In MKN1 cells, an additional pretreatment with the PI3K inhibitor, LY294002, led to the inhibition of pAkt expression and induced apoptosis. The results suggested that SN-38 induces apoptosis by decreasing PI3K-Akt survival signaling, the anti-apoptotic signals, in human gastric cancer cells. Akt inhibitor might be a useful anti-tumor agent in combination with CPT-11.

Key words: apoptosis; gastric cancer; PI3K-Akt pathway; SN-38

Gastric cancer continues to be one of the most common malignancies in Japan. While the development of diagnostic modalities and surgical techniques has improved prognosis, the associated mortality is still the second highest in Japan after lung cancer (Editorial Board of the Cancer Statistics in Japan, 2003). This mortality is due to lack of effective treatment, especially in respect to chemotherapy.

New anticancer agents have recently been used in gastric cancer therapy. Camptothecins are broad-spectrum anticancer drugs that specifically target DNA topoisomerase I (TopoI). The formation of a cleavable drug-TopoI-DNA complex results in lethal double-strand DNA breakage and cell death (Xu and Villalona-Calero, 2002). CPT-11 is a new semisynthetic derivative of camp-

Abbreviations: DMSO, dimethyl sulfoxide;ID_{80}, dose giving 80% inhibition; NF-κB, nuclear factor-κB; pAkt, phospho-Akt; PI3K, phosphatidylinositol-3-OH kinase; PIP_2, phosphatidylinositol-3,4-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TopoI, topoisomerase I; XTT, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate

tothecin that is ultimately converted into the active metabolite SN-38 by carboxylesterase, which is at least 100-fold more cytotoxic than CPT-11 (Rokudai et al., 2002). It has also been reported that exposure to camptothecin derivatives induces apoptosis (Yoshida et al., 1993) and affects the cell cycle by inducing a dose-dependent delay in the S phase followed by dose-dependent trapping in the G2/M phase (Falk and Smith, 1992). However, the precise mechanisms of SN-38-induced apoptosis have not been elucidated.

The phosphatidylinositol-3-OH kinase (PI3K)-Akt signaling pathway is known to transmit survival-promoting signals and to protect a variety of cells from apoptosis (Franke et al., 1997). After growth factor stimulation, PI3K is activated and generates phospholipid second-messenger molecules, phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and phosphatidylinositol-3,4-bisphosphate (PIP_2), which cause a diverse set of cellular responses. One target of PI3K is the serine/threonine kinase Akt [also known as protein kinase B or RAC-PK (Balendran et al., 1999)]. With the generation of PIP_3 and PIP_2 proteins by stimulation with growth factors and cytokines, Akt is recruited to the plasma membrane and is then phosphorylated at two key regulatory sites, Thr308 in the activation loop of the catalytic domain and Ser473 in the COOH-terminal regulatory domain (Alessi et al., 1997). The phosphorylation of Akt at Thr308 is catalyzed by ubiquitously expressed PDK1 (Datta et al., 1997; Le et al., 1998; Stephens et al., 1998). The kinase responsible for phosphorylation of Akt at Ser473 was reported to be PDK2 [PDK1 bound to a fragment of PRK2 (Alessi et al., 1997)]. Phosphorylation at both residues is necessary for full activation of Akt and the subsequent regulation of many cellular processes. Activated Akt phosphorylates proapoptotic Bcl-2 family member Bad, caspase family member, caspase-9, Forkhead family transcription factor, FKHRL1, and IκB kinase, leading to cell survival (Kawato et al., 1991; Cardone et al., 1998; Brunet et al., 1999; Romashkova and Makarov, 1999). Thus, PI3K-Akt signaling pathway inactivation might correlate with apoptosis.

Here, we analyzed SN-38-induced apoptosis in human gastric cancer cell lines. Our data suggest that in some human gastric cancer cells, SN-38 induces apoptosis, which might be partly explained by PI3K-Akt pathway inactivation.

Materials and Methods

Cell culture and reagents

MKN1, MKN28, MKN45, MKN74 and KATO-III cells were purchased from the Riken Cell Bank and grown in RPMI-1640 supplemented with 10% fetal bovine serum, 100 units/mL of penicillin G sodium, 100 μg/mL of streptomycin sulfate and 0.25 μg/mL of amphotericin B equilibrated with 5% CO_2 in air at 37˚C. SN-38 was obtained through Daiichi Pharm (Tokyo, Japan), and LY294002 was purchased from Sigma (St. Louis, MO). SN-38 and LY294002 were dissolved in dimethyl sulfoxide (DMSO). Dilutions with DMSO were made immediately before use to adjust the DMSO concentration in the growth medium to 0.1% for all experiments.

Growth inhibition experiments

The effect of the SN-38 on the proliferation of cells was evaluated using the sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay (Cell Proliferation Kit II, XTT; Roche Molecular Biochemicals, Mannheim, Germany). The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolically active cells. Briefly, cells were seeded into 96-well plates at a density of 2×10^3 viable cells per well in 100 μL medium. After 24 h incubation, a different dose of SN-38 (0–500 nM) was added to quadruplicate wells. Then after 72 h incubation, 50 μL of the XTT was added to each well (final concentration 0.3 mg/mL), followed by further incubation for 4 h in a humidified atmosphere containing 5% CO_2 at 37˚C. The absorbance of the samples was measured at 490 nm using a microplate reader (Wellreader; Seikagaku Corporation, Tokyo, Japan).

The dose giving 80% inhibition (ID_{80}) was calculated in terms of the absorbency in treated cells relative to the absorbency in untreated control cells.

Cell cycle analysis

Cell cycle distribution was analyzed by determining the DNA content and the sub-G1 peak corresponding to the apoptotic cells using flow cytometry. MKN45 (3×10^6) and MKN1 (5×10^5) cells were seeded on 75 cm^2 tissue culture dishes and allowed to grow overnight at 37˚C with 5% CO_2. Cells were exposed to 50 μM LY294002 for 1 h, or not exposed. Cells were next exposed to 20 nM or 250 nM SN-38 for 48 h, then collected, resuspended in 70% ethanol and stored at –20˚C until use. After fixation, cells were incubated for 20 min at 37˚C with RNase A (50 μg/mL) and then for 10 min at room temperature with propidium iodide (50 μg/mL). After samples were filtered through 35-μm nylon mesh, stained cells were analyzed with an FACS Calibur Cytometer (Becton Dickinson, Franklin Lakes, NJ).

Detection of apoptotic cells

Apoptosis was detected morphologically. Trypsinized adherent and floating cells were washed with phosphate buffered saline and pelleted. After fixation with 3.5% formalin solution, apoptotic cells were assessed morphologically by staining with Hoechist 33342, using ultraviolet laser microscope (Optiphoto-2, Nikon, Tokyo).

Western blot analysis

MKN45 and MKN1 cells were treated with LY294002 and/or SN-38 as in the case for cell cycle analysis. The cells were lysed in a sample buffer [Tris-HCl (pH 6.8), SDS, glycerol, bromophenol blue] containing protease inhibitor (complete, mini; Roche Molecular Biochemicals). Following sonication, the total protein was quantified by a BCA method (BCA Protein Assay Kit; Pierce, Rockford, IL). Each boiling sample (60 μg protein/lane) was separated by 10% SDS-PAGE and blotted onto a poly vinilidene difluoride Immobilon membrane. The antibodies used in this study were anti-phospho Akt (Ser473) polyclonal antibody (#9271, Cell Signaling Technology, Beverly, MA), anti-phosphatase and tensin homologue deleted on chromosome 10 (PTEN) polyclonal antibody (#21940, Upstate Cell Signaling Solutions, Charlottesville, VA) and anti-β-actin monoclonal antibody (AC-15, Sigma).

Results

The effect of SN-38 on cell viability and apoptosis in MKN1 and MKN45 cells

We first examined the susceptibility of the two human gastric cancer cell lines, MKN1 and MKN45, to SN-38 treatment. Figure 1 shows the dose-dependent cytotoxic effect of SN-38 against MKN1 and MKN45 cells using an XTT cell proliferation assay kit. The ID_{80}s of MKN45 and MKN1 cells were 20 nM and 250 nM, respectively. We also examined the susceptibility of three other human gastric cancer cell lines, MKN74, MKN28 and KATO-III cells. ID_{80}s of MKN74, MKN28 and KATO-III cells were 15 nM, 200 nM and 30 nM, respectively. MKN45, MKN74 and KATO-III cells showed high sensitivity to SN-38 (ID_{80} < 50 nM), and MKN1 and MKN28 cells showed low sensitivity to SN-38 (ID_{80} > 100 nM). Then we used MKN45 and

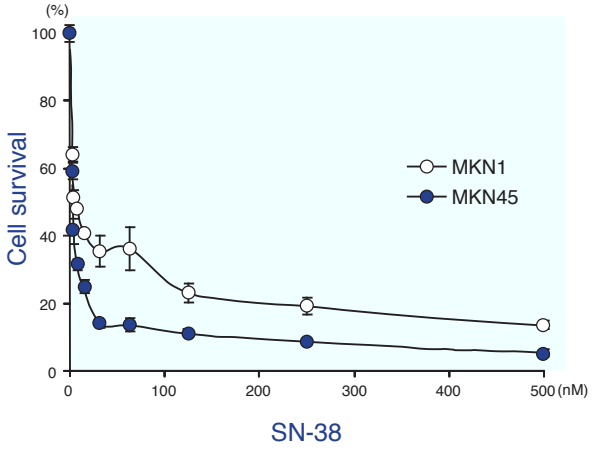


Fig. 1. XTT assay of MKN1 and MKN45 cells treated with SN-38. The rates of MKN1 cells are shown by open circles, and those of MKN45 cells, by closed circles.

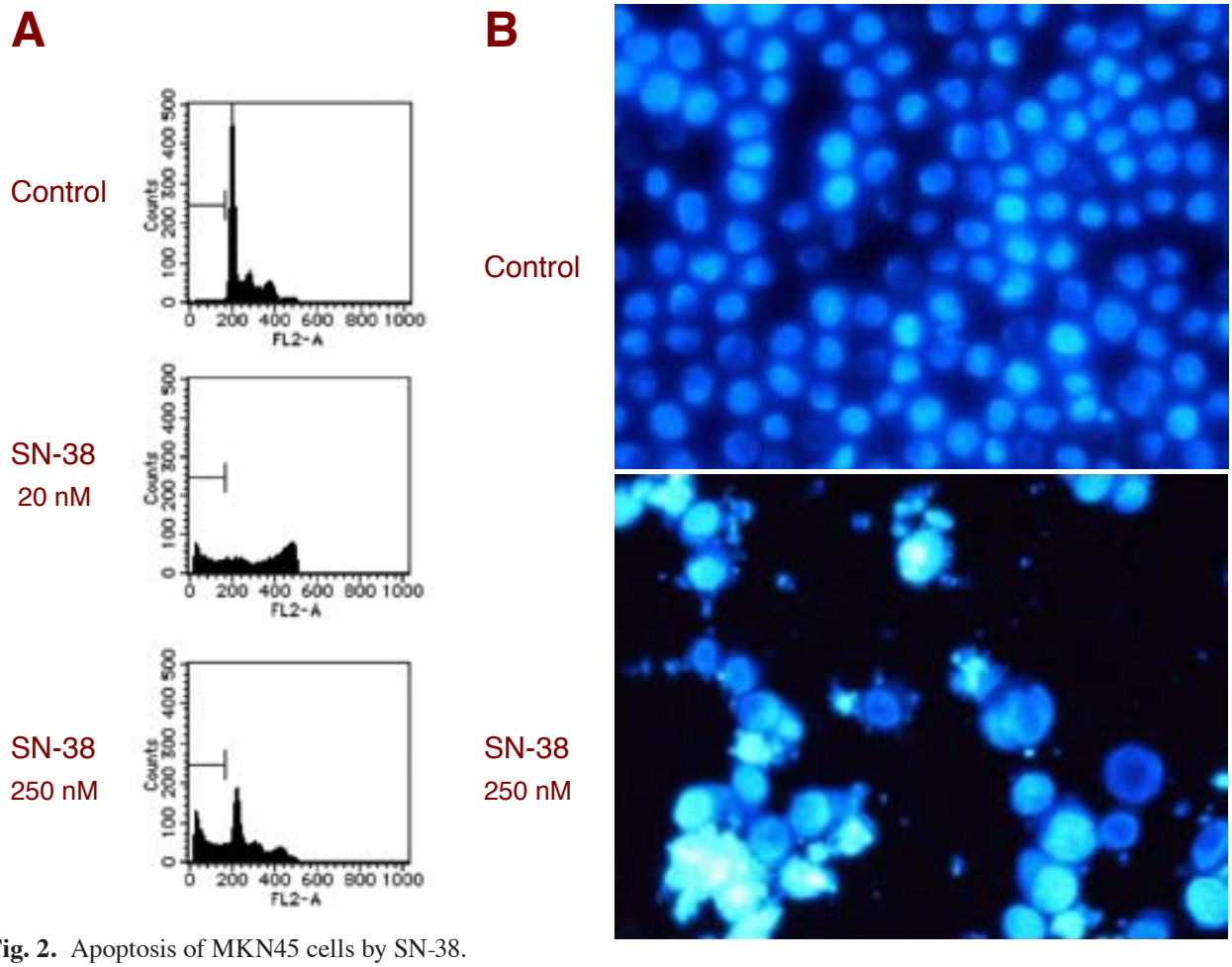


Fig. 2. Apoptosis of MKN45 cells by SN-38.
A: Flow cytometry of MKN45 cells treated with 20 nM or 250 nM SN-38 for 48 h.
B: Fluorescent microscopy of MKN45 cells treated with 250 nM SN-38 for 48 h, and stained with Hoechst 33342 fluorescent dye.

MKN1 cells as the representative cell lines for the following studies.

Then, we examined the effect on the cell cycle in MKN1 and MKN45 cells after SN-38 treatment using flow cytometry. An evident apoptotic sub-G1 fraction was shown in MKN45 cells 48 h after treatment with 250 nM SN-38 (Fig. 2A). We further confirmed SN-38-induced apoptosis of MKN45 cells morphologically by fluorescence microscopy with a Hoechst 33342: 250 nM SN-38 induced apoptosis in MKN45 cells, in which there were condensed and fragmented nuclei (Fig. 2B). However, MKN1 cells treated with 250 nM SN-38 suffered delays in the S phase (Fig. 3A) and had a few apoptotic cells (Fig. 3B). As shown in Fig. 4, the apoptotic sub-G1 fractions of the population of MKN45 and MKN1 cells 48 h after treatment with 250 nM SN-38 were 36.8 and13.5%, respectively.

pAkt and PTEN expression after treatment with SN-38 in gastric cancer cell lines

Next, we examined pAkt and PTEN expression after treatment with SN-38 by Western blotting. In MKN45 cells, the expression levels of pAkt were reduced in a dose-dependent manner by SN-38 (Fig. 5). On the other hand, the expression levels of pAkt were not affected by SN-38 in MKN1 cells. The expression levels of PTEN were not affected in either cell line (Fig. 5).

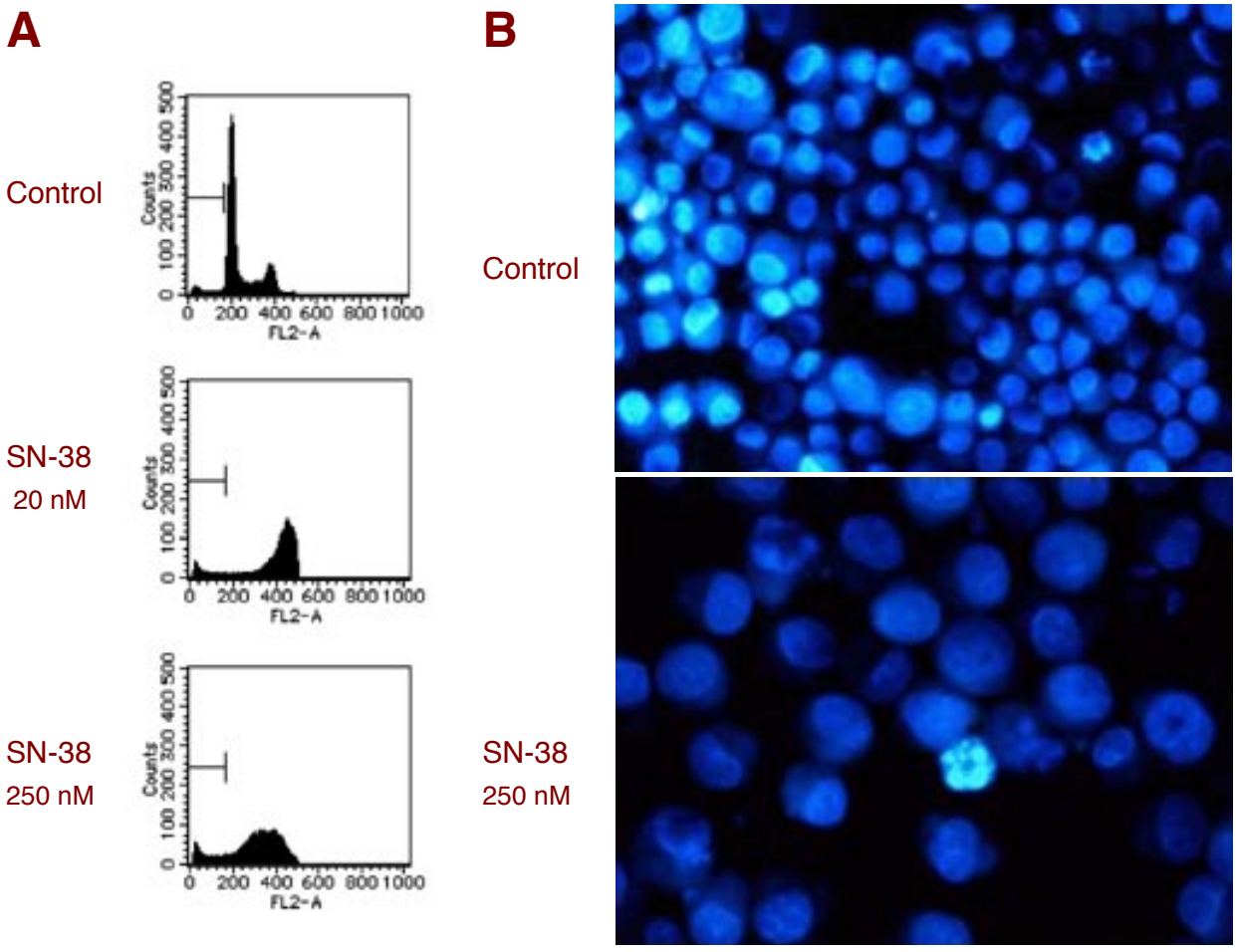


Fig. 3. Influence of SN-38 to MKN1 cells.
A: Effect of 20 nM or 250 nM SN-38 on the cell cycle progression of MKN1 cells after 48 h incubation determined by flow cytometry.
B: Fluorescence microscopy dyeing, Hoechst 33342 after 250 nM SN-38 treatment for 48 h.

PI3K inhibitor reduced the expression level of pAkt and induced apoptosis in MKN1 cells

Through SN-38 treatment, pAkt expression in MKN45 cells was reduced but not in MKN1 cells while MKN45 cells have a higher sensitivity to SN-38 than MKN1 cells. To elucidate that the PI3K-Akt pathway is involved in SN-38-induced apoptosis, we examined whether LY294002 could affect SN-38-induced apoptosis in MKN1 cells. LY294002 is a specific PI3K inhibitor that has been widely used to study the role of PI3K in various biological responses.

The expression level of pAkt in MKN1 cells reduced in an SN-38 dose-dependent manner after

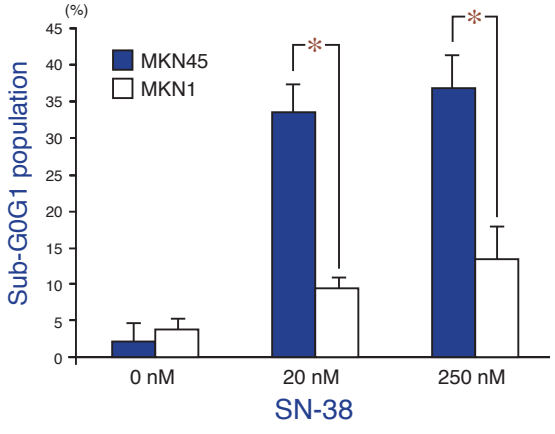


Fig. 4. Sub-G0G1 population in MKN45 and MKN1 cells treated with 0, 20 or 250 nM SN-38 for 48 h. *$P < 0.05$ using Student's *t*-test.

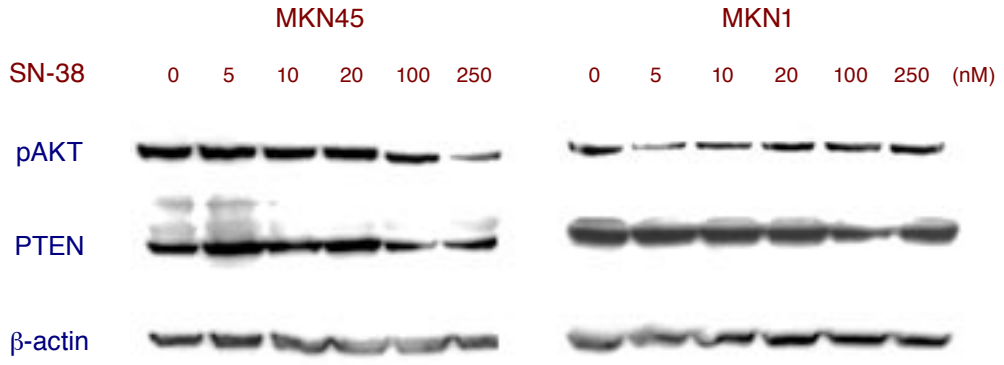


Fig. 5. Western blotting of pAkt and PTEN expression after 0, 5, 10, 20, 100 or 250 nM SN-38 treatment for 48 h.

pretreatment with 50 μM LY294002 (Fig. 6). We also examined PTEN expression using Western blotting, but LY294002 had no influence on PTEN expression (Fig. 6).

Moreover, by cell cycle analysis, the apoptotic sub-G1 fraction appeared by flow cytometry (Fig. 7A). By morphological examination, apoptotic cells were increased by treatment with SN-38 and LY294002 (Fig. 7B). The apoptotic sub-G1 fraction of the population of MKN1 cells was 35.9% 48 h after treatment with 250nM SN-38 and 50 μM LY294002 (Fig. 8). Exposure to 50 μM LY294002 increased SN-38-induced apoptosis in MKN1 cells to as much as that in MKN45 cells after treatment with only SN-38 (Fig. 8). These results indicated that the PI3K-Akt pathway plays a significant role in SN-38-induced apoptosis in human gastric cancer cells.

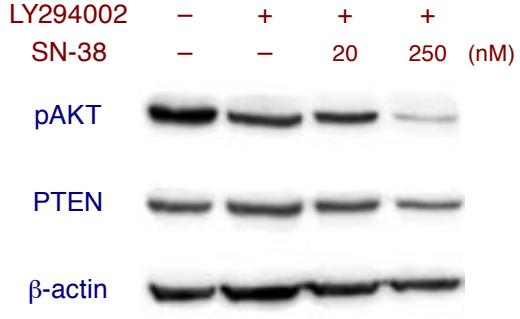
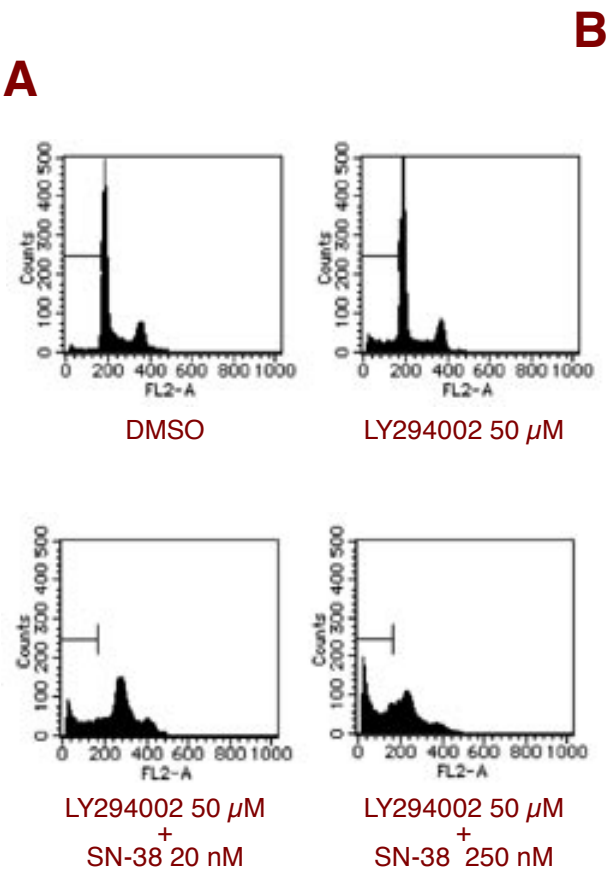


Fig. 6. Western blot of pAkt and PTEN expression. MKN1 cells were treated with 0, 20 or 250 nM SN-38 for 48 h in the presence of 50 μM LY294002.

Discussion

The MKN45 cells were more sensitive to SN-38 than the MKN1 cells. The difference of sensitivity mainly comes from the percentage of apoptotic cells. The pattern of tumor response after in vivo topotecan treatment was reported to correlate with the ability of the drug to induce apoptosis but not with its in vitro antiproliferative activity (Caserini et al., 1997). Apoptosis is a major mode of cell death in response to chemotherapeutic drug treatment (Hickman, 1992; Kerr et al., 1994), and resistance to apoptosis induction has been proposed as a critical mechanism in drug resistance (Kataoka et al., 1994; Chen et al., 1996; Seimiya et al., 1997). However, the mechanisms by which chemotherapeutic agents induce apoptosis are not fully understood. The sensitivity of cells to chemotherapeutic drug-induced apoptosis appears to depend on the balance between proapoptotic and antiapoptotic signals. Therefore, it is possible that a chemotherapeutic drug may induce apoptosis not only by increasing the proapoptotic signals but also by decreasing the antiapoptotic ones.

The PI3K-Akt signaling pathway is known to transmit survival signals and to protect a variety of cells from apoptosis. Akt plays a critical role in controlling the balance between cell survival and apoptosis (Balendran et al., 1999). Phosphorylation of Akt is promoted by phosphatidylinositides converted by PI3K products. Previous reports have



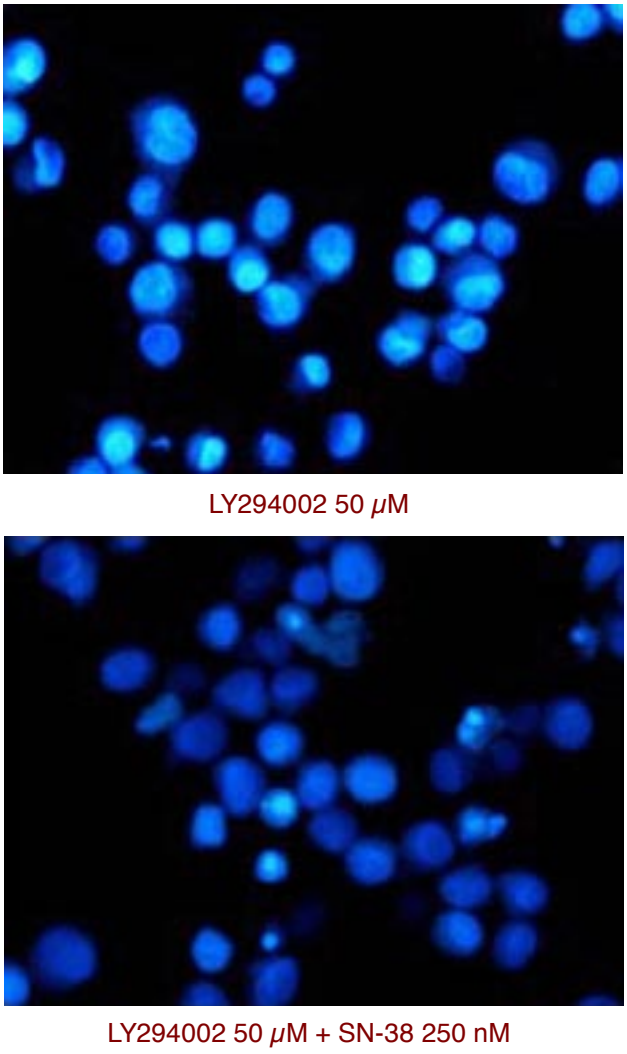


Fig. 7. Influence of LY294002 to MKN1cells.
A: Cell cycle analysis by flow cytometry.
B: Morphological detection of apoptosis by Hoechst 33342 staining.

shown that Akt delivers antiapoptotic survival signals by phosphorylating Bad and activating caspase-9 (Cardone et al., 1998; Brunet et al., 1999). In addition, inhibition of Akt signaling can induce apoptosis in some human cancer cell lines (Page et al., 2000; Yuan et al., 2000). It has also been reported that Akt plays an important role in cell survival when cells are exposed to different apoptotic stimuli, such as growth factor withdrawal, ultraviolet radiation, matrix detachment, cell cycle discordance and DNA damage (Hemmings, 1997; Kulik et al., 1997; Kulik and Weber, 1998; Ng et al., 2000; Brognard et al., 2001). Moreover, inactivation of PI3K using a specific inhibitor has led to dephosphorylation of Akt at Ser473, consequently causing translocation of Akt to nuclei,

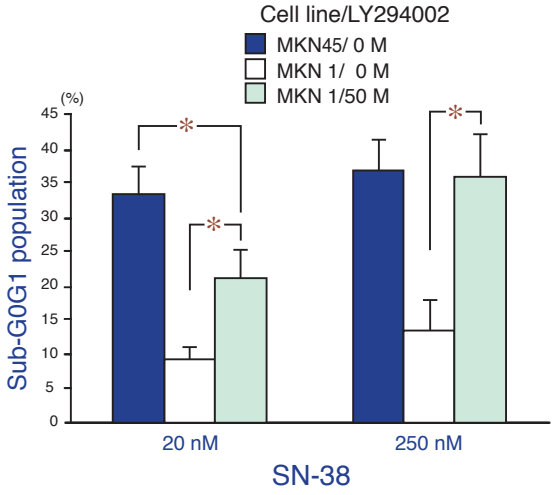


Fig. 8. Sub-G0G1 population in MKN45 and MKN1 cells treated with 0, 20 or 250 nM SN-38 in the presence or absence of LY294002 for 48 h. *$P < 0.05$ using Student's *t*-test.

where it is believed to regulate the transcription of genes mediating cell survival or apoptosis (Hemmings, 1997). In a previous study, TRAIL and Fas induced apoptosis by inhibition of the PI3K-Akt pathway in human gastric cancer cells (Nam et al., 2003; Osaki et al., 2004). Our study showed that apoptosis was induced in cells where pAkt expression was reduced (MKN45), but not in cells where pAkt expression showed no change (MKN1) by SN-38. It has been reported that exposure to camptothecin induced a dose-dependent trapping in the G2/M phase affects and dose-dependent delay in the S phase. Treatment of MKN1 cells with 48 h 20 nM SN-38 induced G2/M phase arrest, and 48 h 250 nM SN-38 treatment induced S phase delay. Meanwhile in MKN45 cells, 48 h 20 nM SN-38 treatment induced G2/M phase arrest tendency and 48 h 250 nM SN-38 treatment induced sub-G1 peak. We speculate that low dose SN-38 treatment below 20 nM induces the G2/M arrest in MKN45 cells and 20 nM SN-38 treatment begins to induce S phase cells. Moreover, in MKN45 cells, 250 nM SN-38 treatment induces apoptosis by reducing pAkt expression but MKN1 cells showed resistance for apoptosis by no change in pAkt expression.

Therefore, inactivation of pAkt using the PI3K inhibitor, LY294002, finally induced apoptosis in MKN1 cells as well as in only SN-38-treated MKN45 cells. It was also reported (Nakashio et al., 2000) that constitutively active Akt results in a reduction of the cytotoxic effect of topotecan in lung carcinoma cells. The PI3K-Akt pathway might thus play a significant role in SN-38-induced apoptosis in human gastric cancer cells.

The tumor suppression gene PTEN is a natural biological inhibitor of Akt cell survival signals. PTEN expression sensitizes cells to death receptor-mediated apoptosis induced by a variety of stimuli, such as tumor necrosis factor (Mayo et al., 2002), TRAIL (Thakkar et al., 2001) and chemotherapeutic agents (Tanaka et al., 2000). Akt is abnormally activated in several human tumors by loss of PTEN (Di Cristofano and Pandolfi, 2000; Simpson and Parsons, 2001). Recent studies have documented that PTEN appears to negatively regulate PI3K-Akt survival signaling by removing the D-3 phosphate from PIP_3, which is a primary activator of Akt (Maehata and Dixon, 1998). Loss of PTEN results in high basal activity of Akt in a variety of tumors, while introduction of PTEN suppresses the activity. It is reported (Saga et al., 2002) that overexpression of PTEN increased sensitivity to SN-38 in ovarian cancer cells. However, in our study, the expression of PTEN was not correlated with SN-38-induced apoptosis; in human gastric cancer cells, SN-38 might suppress the PI3K-Akt signaling via a PTEN independent pathway. Rokudai et al. (2002) reported that suppression of PDK1, but not of PI3K, was one of the mechanisms of chemotherapeutic drug-mediated Akt inactivation, because VP-16 indirectly suppressed PDK1 kinase activity in 293T cells. However, the mechanisms of SN-38-mediated Akt inactivation remain unclear. Overexpression of constitutive active Akt using a transfected active form of *akt* cDNA overcame chemotherapeutic drug-induced apoptosis. Akt inactivation might be a sign of chemotherapeutic drug sensitivity of cancer cells, because it was found only in chemosensitive cells (MKN45).

Nuclear factor-κB (NF-κB) is a ubiquitously expressed transcription factor involved in a wide spectrum of cellular functions like cell cycle control, stress adaptation, inflammation and control of apoptosis (Pahl, 1999). NF-κB is constitutively activated in various types of tumors, including gastric cancer (Sasaki et al., 2001). Several investigators have reported that inhibition of the NF-κB pathway by adenoviral delivery of an IκBα superrepressor, small interfering RNA, against the p65 subunit of NF-κB or TNFα increased SN-38-induced apoptosis (Camp et al., 2004; Guo et al., 2004; Valente et al., 2003). Besides the PI3K-Akt pathway, the NF-κB pathway might also facilitate an antiapoptotic signal to inhibit SN-38-induced apoptosis.

In conclusion, we found that SN-38 induced apoptosis by decreasing the PI3K-Akt survival signaling, the antiapoptotic signals in human gastric cancer cells. A selective small molecule inhibitor of Akt, the Akt/protein kinase B signaling inhibitor-2 (API-2) was recently discovered by screening the National Cancer Institute Diversity Set (Yang et al., 2004). Such an Akt inhibitor might be a useful

anti-tumor agent in combination with a chemotherapeutic agent, such as CPT-11.

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