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

Shizue Yoshioka, Shigeru Tatebe, Naruo Tokuyasu, Kenjiro Taniguchi, Yoshimi Ogami, Osamu Yamamoto, Keigo Ashida, Yoshihito Gomyo, Akira Kondo, Shunichi Tsujitani and Masahide Ikeguchi

Division of Surgical Oncology, Department of Surgery, School of Medicine, Tottori University Faculty of Medicine, Yonago 683-8504 Japan

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

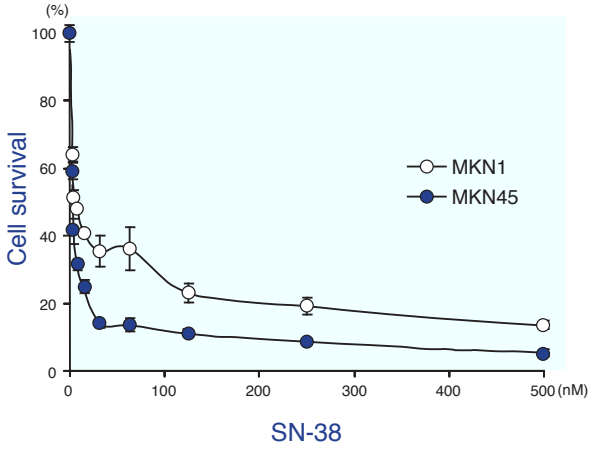

Fig. 1. XTT assay of MKN1 and MKN45 cells treated with SN-38. The rates of MKN1 cells are shown by open circles, and those of MKN45 cells, by closed circles.

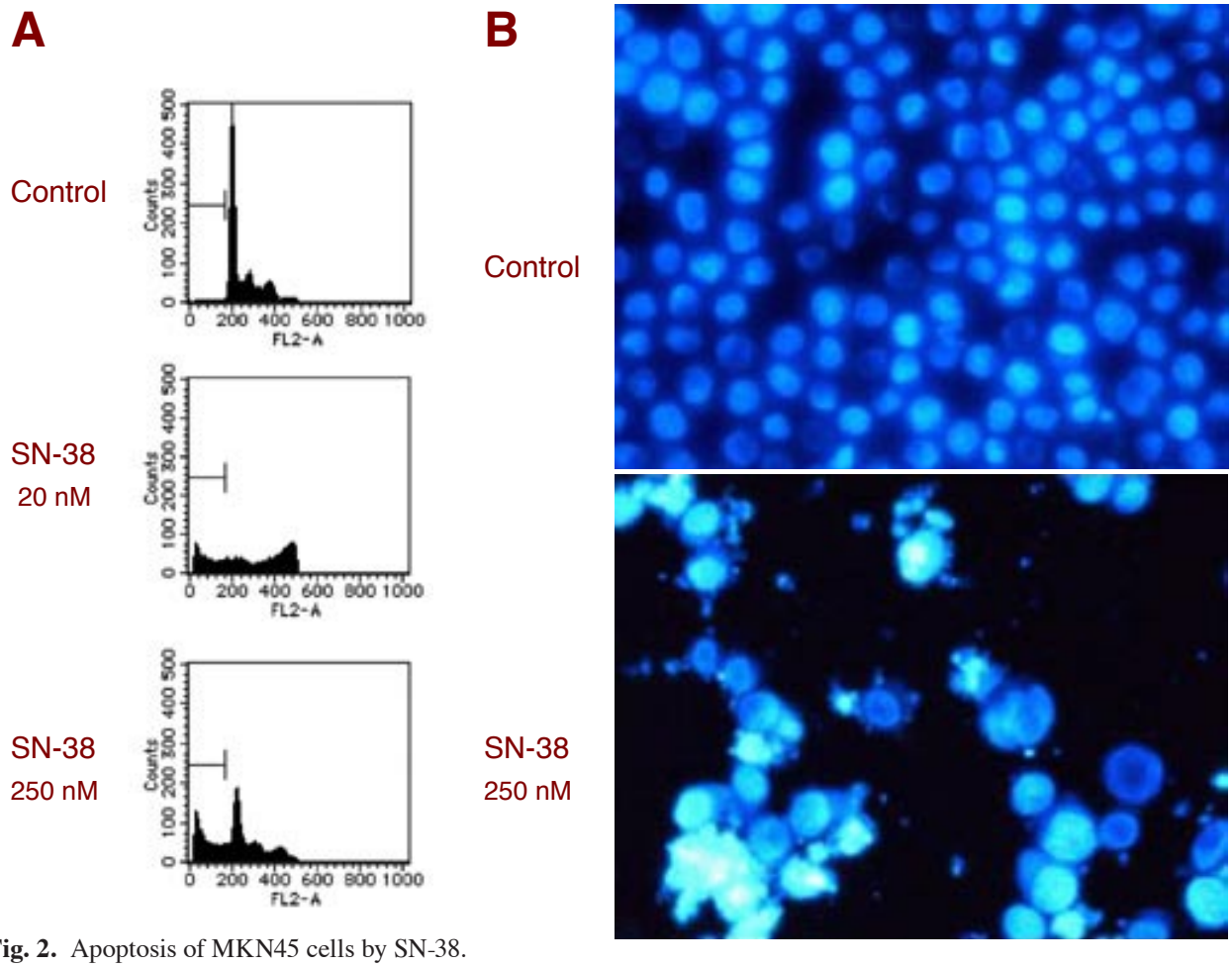

Fig. 2. Apoptosis of MKN45 cells by SN-38.
A: Flow cytometry of MKN45 cells treated with 20 nM or 250 nM SN-38 for 48 h.
B: Fluorescent microscopy of MKN45 cells treated with 250 nM SN-38 for 48 h, and stained with Hoechst 33342 fluorescent dye.

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).