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Full Paper

Irinotecan Activates p53 With Its Active Metabolite, Resulting in Human Hepatocellular Carcinoma Apoptosis

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Abstract. The topoisomerase I inhibitor irinotecan is widely used in anticancer therapy, although the detailed mechanism is still unclear. We investigated the apoptotic mechanisms of irinotecan in human hepatocellular carcinoma (HCC) cell lines (Huh7). SN-38 caused a significant decrease in cell proliferation and induced apoptosis in Huh7 cells and HepG2 cells. SN-38 significantly increased the expression of p53 protein and its phosphorylation at Ser^{15} in the nucleus and apoptosis-inducing proteins Bax, caspase-9, and caspase-3, while it significantly decreased the antiapoptosis protein Bcl-xL of Huh7 cells. SN-38-induced apoptosis was recovered after *p53* antisense oligodeoxynucleotide (AS ODN) pretreatment, while Huh7 cells were precultured with *p53* AS ODN, followed by the addition of SN-38 for 24 h. Furthermore, increases in *p53* DNA-binding activity were observed in the nuclei of Huh7 cells after SN-38 treatment as shown by electrophoretic mobility shift analysis. SN-38 binding motifs were detected in the proximal promoter of *p53* (bases –433 to –317 and –814 to –711). These results suggest that the p53-mediated apoptosis pathway is important in the anticancer effects of irinotecan in hepatocellular carcinoma.

Keywords: irinotecan, SN-38, Huh7 cell, apoptosis, *p53* gene

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers. HCC occurs in chronic liver diseases associated with persistent infection with hepatitis B or C virus in the majority of the cases (1, 2). Although surgery is considered the best option treatment, unfortunately surgery is contraindicated in a majority of patients with HCC at diagnosis. Therefore, nonsurgical therapy is important from the viewpoint of developing effective chemotherapy agents for the treatment of HCC. Anticancer drugs such as doxorubicin, camptothecin, and irinotecan target topoisomerase to cause DNA damage to cancer cells. These drugs have been used in first-line chemotherapy in patients with colorectal, esophageal, and lung cancer (3, 4). Irinotecan is an anticancer prodrug activated by esterases producing the active metabolite SN-38 (7-ethyl-10-hydroxy-camptothecin), which has at least 1000-fold more potent anticancer activity than irinotecan. Stabilization of a cleavable complex with irinotecan treatment is accompanied by G2/M cell-cycle arrest and apoptosis in colon cancer (5). Apoptosis induced by irinotecan or SN-38 is associated with cleavage of poly (ADP-ribose) polymerase (PARP) in colon carcinoma cells (6). SN-38 also shows anticancer activity against a wide range of tumors (3, 7, 8). SN-38 arrests the cell cycle in G2 and induces cell death via promotion of Bcl-2 family and p53 protein in testicular cancer (9). The *p53* tumor suppressor gene, which is the most common genetic alteration in human cancer, encodes a short-lived transcription factor (10). In response to a variety of stresses to the cell, the posttranslational modification of p53 is known to inhibit cell growth and apoptosis (10 – 12). Recent studies have indicated that *p53* gene transfection increases the chemosensitivity to specific anticancer drugs of human liver tumor and several other types of tumor cells (13, 14). Many molecular targets

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of these drugs have been suspected to mediate p53-dependent apoptosis via activation of Bax and down-regulation of Bcl-xL. In addition, cell death depends on amplification of the death receptor signals via the mitochondria and occurs as a direct consequence of ligation of the death receptor with activation of a downstream procaspase such as caspase-3 (15, 16). However, the molecular mechanisms behind the therapeutic effects of irinotecan and SN-38 in hepatic cancer have not yet been fully elucidated.

We therefore examined the detailed mechanisms of apoptosis by SN-38 in a HCC cell line (Huh7) derived from Japanese patients and differentiated human liver cancer functions (17, 18), identified novel SN-38 responsive regions within the promoter region of *p53*, and attempted to explain how this apoptosis is mediated by p53 in HCC.

Materials and Methods

Drug and antibodies

SN-38 was obtained from Daiichi Pharmaceutical Co., Ltd. (Tokyo). The drug was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C until use. Antibodies used in the present study were anti-p53 polyclonal antibody (Ab) (Chemicon International, Inc., Temecla, CA, USA); anti-phosphorylated serine residue (Ser^{15}) p53 Ab, anti-caspase-3 Ab, anti-caspase-9 polyclonal Ab, anti-Bcl-xL polyclonal Ab, and anti-Bax polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell lines and cell cultures

The HCC line Huh7 with mutated p53 (a point mutation of codon 220) and HepG2 carrying wild-type p53 as a control were obtained from the Riken Gene Bank (Tsukuba). The cells were cultured with RPMI-1640 medium (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), with penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C under an atmosphere of 5% CO_2 and 95% air. The cells were seeded at 5.0×10^5 cells in culture dishes. The cells were incubated for 48 h, and the second day was designated as day 0. From day 0, the cells were incubated with SN-38 at a concentration of 12.5, 25, or 50 ng/ml for 24 h. The SN-38 concentration of 25 ng/ml is the peak plasma concentration that can clinically inhibit tumor growth in cancer (19, 20).

DNA fragmentation

DNA fragmentation was analyzed using the Apoptotic DNA-Ladder kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. Briefly, the cells (5×10^5) were incubated with SN-38 for 24 h. After incubation, the cells were lysed using a lysis buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100, pH 4.4). DNA was extracted using a filter system with glass fiber fleece. DNA samples were loaded onto a 2% agarose gel column operated at 100 V and then stained with ethidum bromide (0.5 mg/ml) for 20 min. DNA fragmentation was visualized under UV light.

Terminal dUTP nick-end labeling (TUNEL) assay

Huh7 and HepG2 were cultured on microchamber slides (Becton Dickinson Labware, Bedford, MA, USA) and allowed to adhere overnight. They were treated with SN-38 for 12, 24, and 48 h. Furthermore, they were preincubated with *p53* sense and antisense oligodeoxynucleotide (SE ODN and AS ODN, respectively, 5 μM each) for 3 h, followed by the addition of SN-38 for 24 h. Apoptosis of the cells was evaluated based on the TUNEL assay using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA). The cell samples were fixed with 4% paraformaldehyde for 25 min and permeabilized with 0.2% Triton X-100 for 5 min. Endogenous peroxidase was quenched in 0.3% hydrogen peroxide. Diaminobenzidine and hematoxylin were used as the chromogen and the counterstain, respectively.

Cell proliferation assay

Cell proliferation was evaluated in the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) cell-titer 96 aqueous one-solution cell proliferation assay (Promega) according to the manufacturer's instructions. The cells were incubated with SN-38 in 96-well plates (Iwaki Glass, Tokyo) for 24 h. The MTS substrate was added to each well in a ratio of 20 μl of MTS for every 100 μl of culture media, and the mixture was incubated for 2 h at 37°C. Absorbance was measured at 490 nm with a microplate reader (Multiskan, Thermo Labsy-sytems, Ventaa, Finland). The cell proliferation rate was expressed as the ratio of optical density of the treated to control cells. At least 8 independent experiments were performed for each experimental condition.

p53 AS ODN and cell culture

The SE sequence (5'-TCACTGCCATGGAGGA-3') and AS sequence (5'-TCCTCCATGGCAGTGA-3') ODNs (5 μM each) specific for *p53* were designed (Gene Bank No. X02469) and synthesized in phosphorothiolated form. Huh7 and HepG2 cells were preincubated in the 3 or 5 μM of ODNs for 3 h each. There-

after, SN-38 was added to each sample.

Western blot analysis

The nuclear proteins in the cells were prepared using a commercial kit (NE-PER nuclear and cytoplasmic extraction kit; Pierce Biotechnology, Rockford, IL, USA), and whole proteins were also extracted with cell lysing buffer (0.1% NP-40 in PBS). The protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equivalent amounts of proteins (25 μg) were resolved by SDS-PAGE using 10% polyacrylamide gels. Thereafter, proteins were transferred onto nitrocellulose membranes (Amersham Bioscience, Buckinghamshire, UK), and blocked with 5% skim milk with Tween-Tris-buffered saline (TTBS) (150 mM NaCl, 100 mM Tris, pH 7.5, and 0.05% Tween 20) for overnight at 4°C. The blots were probed with specific primary Abs for 2 h at the appropriate dilution. After washing in TTBS, the blots were conjugated with horseradish peroxidase-conjugated second Ab IgG. Reactive proteins were detected in the chemiluminescence assay (ECL plus kit, Amersham Bioscience). The intensity of the detected bands was analyzed using gel-blotting macros in NIH image 1.65 software (Ver. 1.65; National Institutes of Health, Bethesda, MD, USA) (21).

Electrophoretic mobility shift analysis (EMSA)

For EMSA, we used a *p53* probe consisting of a synthetic double-stranded oligonucleotide containing the *p53* proximal promoter region. In brief, 10,000 cpm of [γ-^{32}P]-labeled DNA fragments was incubated at room temperature for 30 min with 20 μg of extracted nuclear proteins in Huh7 and HepG2 cells (expressing wild-type p53), and 2 μg of poly (dI-dC) in reaction buffer (20 mM HEPES-NaOH, 1 mM dithiothreitol, 0.3 mM EDTA, 0.2 mM EGTA, 80 mM NaCl, 10% glycerol, and 0.2 mM PMSF, pH 7.9). DNA-protein complexes were separated from free probe on 6% polyacrylamide gels in Tris-borate buffer containing 0.5 mM EDTA, pH 8.3. Electrophoresis was carried out at 100 V for 2 h at 4°C. The gel was dried and exposed to X-ray film (FujiFilm, Tokyo) with an intensifying screen at −80°C (22).

DNA probes

The probes were derived from sequences present in the p53 promoter region (Gene Bank No. J04238). The p53 proximal promoter was amplified from human genomic DNA by PCR with ODNs: Forward (F): (−195/+1) 5'-TTCCATCAA-3' and Reverse (R): 5'-TCTGAAGCCTGGAGAATG-3', (−297/−94) F: 5'-GTCCGCGGTAATTCTTA-3' and R: 5'-GTTGTATTC CTGAGTGCC-3', (−433/−228) F: 5'-CGAGCTCTT ACTTGCTAC-3' and R: 5'-CCTAACGTTTTCTCC CAG-3', (−562/−308) F: 5'-CCTCATAAGGCTTACG-3' and R: 5'-GAGGAATCCTGCATTGTG-3', (−741 /−484) F: 5'-AAGAAAGGATCCAGCTGA-3' and R: 5'-GTCGCCATGACAAGTAAG-3', (−814/−594) F: 5'-GCTCTGGCTTGCAGAATT-3' and R: 5'-AGTCTT GAGCACATGGGA-3', (−856/−652) F: 5'-GAGAAT CCTGACTCTGCA-3' and R: 5'-CGGTGGCTCTAG ACTTTT-3', (−260/−228) F: 5'-CGAGCTCTTACTT GCTAC-3'and R: 5'-GTTGTATTCCTGAGTGCC-3', (−317/−260) F: 5'-GGCACTCAGGAATACA-3' and R: 5'-CATGGAAACGTAAGCC-3', (−371/−308) F: 5'-CCTCATAAGGCTTACG-3' and R: 5'-CTCCTTCAC AACCCTT-3', (−433/−362) F: 5'-CAGAGTGATAAG GGTTG-3' and R: 5'-CCTAACGTTTTCTCCCAG-3', (−651/−594) F: 5'-GCTCTGGCTTGCAGAATT-3' and R: 5'-AGAGTCAGGATTCTC-3', (−721/−654) F: 5'-GAATCCTGACTCTGCA-3' and R: 5'-CATGACAAG TAAGGGC-3', and (−814/−721) F: 5'-CGGATTACT GCCCTT-3' and R: 5'-AGTCTTGAGCACATGGGA-3'.

Statistical analysis

Data are expressed as the mean ± S.E.M. Differences between groups were analyzed using two-way ANOVA, followed by comparison with the Mann-Whitney U-test or Fisher's PLSD test. A P value of less than 0.05 was considered to represent a statistically significant difference.

Results

SN-38 induced apoptosis in Huh7 and HepG2 cells

We first examined whether SN-38 induced apoptosis in Huh7 and HepG2 cells. The cells incubated with SN-38 for 24 h showed significant, dose-dependent DNA fragmentation (Fig. 1A). In addition, apoptosis in SN-38-treated cells was analyzed using TUNEL staining. In control Huh7 cells, apoptotic cells comprised 4.25 ± 1.32% of the total, but the proportion increased significantly with the SN-38 concentrations of 12.5, 25, and 50 ng/ml (to 18.00 ± 1.73%, 32.00 ± 2.13%, and 38.00 ± 3.22%, respectively, P<0.01) (Fig. 1B). Furthermore, In control HepG2 cells, apoptotic cells comprised 3.05 ± 0.37% of the total, but the proportion increased significantly with the SN-38 concentrations of 12.5, 25, and 50 ng/ml (to 12.03 ± 1.18%, 17.25 ± 1.87%, and 24.63 ± 3.02%, respectively, each P<0.01) (Fig. 1B). Apoptosis after incubation of Huh7 cells with SN-38 25 ng/ml showed a continuous increase of up to 5 – 6-fold compared with control values at 48 h (Fig. 1C). SN-38 at 25 ng/ml also increased apoptosis of HepG2 cells in a time-dependent manner (Fig. 1C).

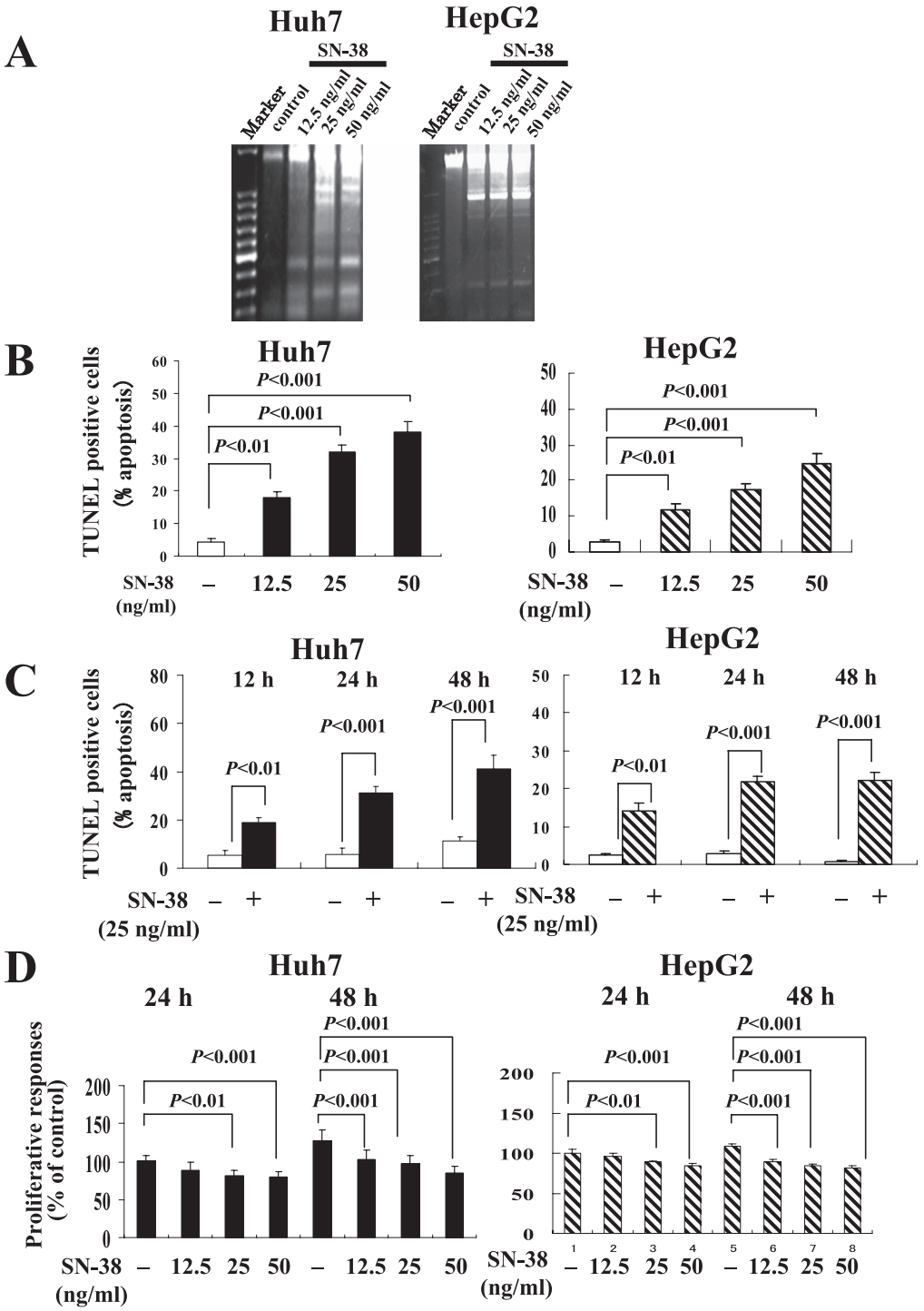


Fig. 1. Effects of SN-38 on apoptosis in Huh7 and HepG2 cells. A: DNA fragmentation. The cells (2×10^4 cells) were cultured for 24 h in the presence of SN-38 at 12.5, 25, and 50 ng/ml, respectively. The data are representative of 3 independent experiments. DNA was isolated and analyzed as described in Materials and Methods. B: TUNEL-positive cells were counted and are shown as the percentage of apoptosis in Huh7 and HepG2 cells treated with different doses of SN-38. Each bar indicates a mean ± S.E.M. The data are representative of 8 independent experiments. Details are described in Materials and Methods. C: The cells were cultured with SN-38 25 ng/ml for 12, 24, and 48 h. TUNEL-positive cells were counted and are shown as the percentage of apoptosis. Each bar indicates a mean ± S.E.M. The data are representative of 8 independent experiments. D: The cells (5×10^4) were cultured with SN-38 in 96-well plates for 24 or 48 h. Cell proliferation was detected in the MTS assay. MTS was added to the medium for an additional 2 h. Cell survival was detected by measuring absorbance at 490 nm. Cell proliferation is expressed as the ratio of the optical density of SN-38-treated cells to that of the controls. Each bar indicates a mean ± S.E.M. The data are representative of 8 independent experiments. Details are described in Materials and Methods.

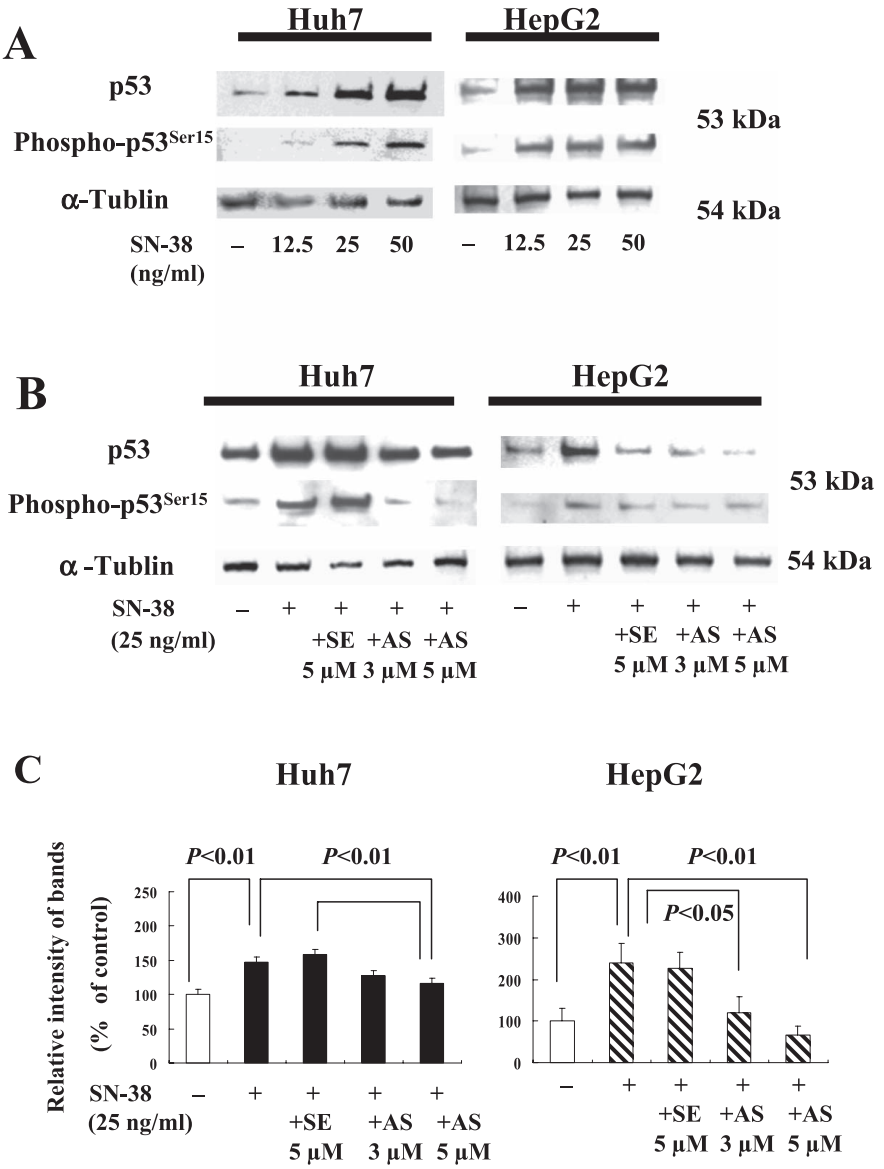


Fig. 2. SN-38 increased expression and phosphorylation of p53 protein in the nuclei of Huh7 and HepG2 cells. A: The cells were cultured for 24 h in the presence of SN-38 12.5, 25, and 50 ng/ml, respectively. The p53 protein expression and Ser^{15} phosphorylation in the nuclear fraction of Huh7 and HepG2 cells treated with SN-38 were determined using Western blotting. α-Tubulin was used as the loading control. The data are representative of 6 independent experiments. B: The cells were precultured for 24 h with SE ODN (5 μM) and AS ODN (3 and 5 μM), followed by the addition of SN-38 25 ng/ml. The p53 protein expression and phosphorylation were determined using Western blotting. α-Tubulin was used as the loading control. The data are representative of 6 independent experiments. C: Western blotting of band intensities of p53 protein expression. Each bar indicates a mean ± S.E.M. The data are representative of 6 independent experiments.

On the other hand, Huh7 and HepG2 cell proliferation, as assessed in the MTS assay, was significantly inhibited after incubation with SN-38 at 25 ng/ml for 24 and 48 h in a dose- and time-dependent manner (Fig. 1D).

Effects of SN-38 on p53 protein expression in the nucleus of Huh7 and HepG2 cells

Western blot analysis demonstrated a significant dose-dependent increase in total p53 protein expression in the nuclear fraction of SN-38-treated Huh7 and HepG2 cells (Fig. 2A). p53 provided protection effects against tumor growth. The important function of activated p53 is critically dependent on the phosphorylation of p53 protein at the Ser^{15} residue in liver cancer (23, 24). Incubation with SN-38 (12.5 – 50 ng/ml) resulted in a dose-dependent increase in the basal expression of p53 protein as well as increase in its phosphorylation at

Ser[15] (Fig. 2A). Furthermore, we investigated the effects of pretreatment with *p53* AS ODN on SN-38-induced apoptosis and p53 expression in the nucleus of Huh7 and HepG2 cells. Increases in p53 expression and phosphorylation at Ser[15] were suppressed by *p53* AS ODN (5 μM) pretreatment (Fig. 2: B and C). The same effects were also seen in the SN-38-treated HepG2 cells. Thus, SN-38 appeared to be effective in both cell lines, indicating that functions of p53 expression and activation have same levels. These results suggest that p53 is key factor in the regulation of SN-38-induced apoptosis in HCC.

Effects of apoptosis on SN-38-treated Huh7 cells in the presence of p53 AS ODN

We examined the apoptosis mechanisms of Huh7 cells treated with SN-38. To examine whether SN-38-induced apoptosis is mediated through the p53-dependent pathway, Huh7 cells were precultured with *p53* AS ODN and SE ODN for 3 h followed by incubation with SN-38. DNA fragmentation was significantly reduced in SN-38-treated cells after pretreatment with *p53* AS ODN at 5 μM (Fig. 3A). Apoptosis was significantly inhibited in Huh7 cells incubated with SN-38 in the presence of *p53* AS ODN compared with the cells incubated with SN-38 alone (SN-38 *vs* SN-38 + *p53* AS ODN: 36.33 ± 2.31% *vs* 24.33 ± 2.40%, respectively, $P<0.05$) (Fig. 3B). Incubation with SN-38 after *p53* SE ODN pretreatment was confirmed to have no effect on apoptosis in this experimental system.

On the other hand, *p53* AS ODN reversed the inhibition of cell proliferation by SN-38 treatment (SN-38 *vs* SN-38 + *p53* AS ODN: 73.98 ± 2.24% *vs* 87.03 ± 2.11%, respectively) (Fig. 3C).

Effects of SN-38 on apoptosis-related protein expression in Huh7 cells

Western blotting showed that SN-38 significantly decreased the expression of the apoptosis-inhibitory protein Bcl-xL (76.50 ± 3.76%, $P<0.05$) in the cytoplasm of Huh7 cells compared with the control cells (100.00 ± 5.70%), while it significantly increased the apoptosis-facilitating proteins Bax (186.70 ± 30.33% *vs* 100.00 ± 17.13% in controls, $P<0.05$), caspase-9 (168.90 ± 6.70% *vs* 100.00 ± 14.18% in controls, $P<0.05$), and caspase-3 (127.74 ± 7.24% *vs* 100.00 ± 10.53% in controls, $P<0.05$) in the cytoplasm of Huh7 cells. Furthermore, *p53* AS ODN reversed the inhibition of Bcl-xL (107.14 ± 9.76%, $P<0.01$) and increased the expression of Bax (99.59 ± 22.22%, $P<0.01$), caspaese-9 (97.32 ± 17.55%, $P<0.05$), and caspase-3 (91.54 ± 7.58%, $P<0.01$) (Fig. 4). These results suggest that SN-38 increases the apoptosis via the mitochondrial pathway.

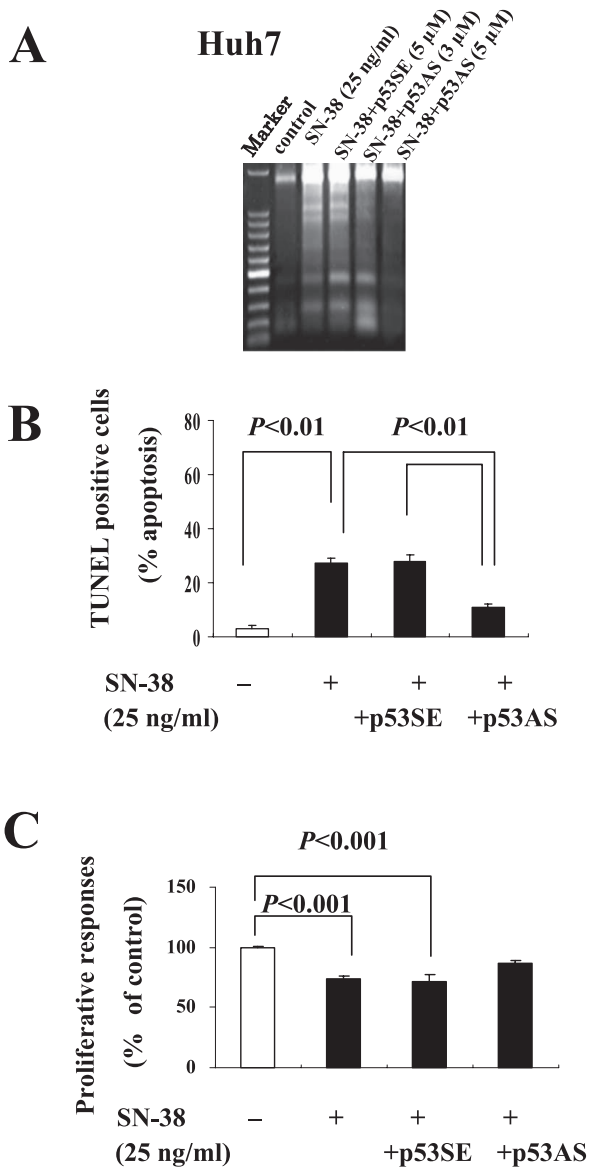


Fig. 3. Effects of *p53* AS ODN on SN-38-induced apoptosis and cell proliferation in Huh7 cells. Huh7 cells were pretreated with *p53* AS ODN (5 μM) or SE ODN (5 μM) followed by 24 h incubation with SN-38. A: DNA fragmentation was observed in SN-38-treated cells in the presence of *p53* AS ODN (3 and 5 μM). B: Cell apoptosis was analyzed based on the number of TUNEL-positive cells in histologic analysis. Each bar indicates a mean ± S.E.M. The data are representative of 6 independent experiments. C: The cell proliferation rate was measured in the MTS assay as described in Materials and Methods. Each bar indicates a mean ± S.E.M. The data are representative of 8 independent experiments. Details are described in Materials and Methods.

p53 SE ODN did not affect the change in apoptosis-related protein expression after SN-38 treatment.

Effect of SN-38 on the DNA-binding activity of p53 in Huh7 cells

We examined the p53 DNA-binding activity in

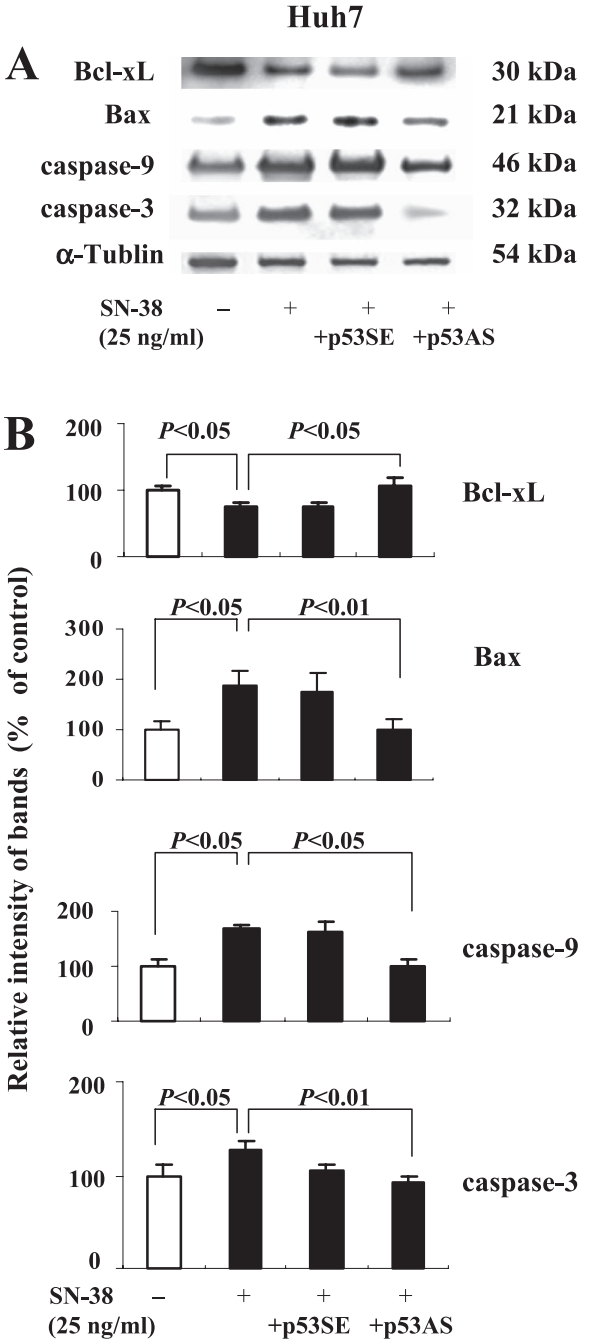


Fig. 4. Expression of apoptosis-related proteins in Huh7 cells after SN-38 treatment. The cells were pretreated with *p53* AS ODN (5 μM), followed by 24 h incubation with SN-38. Apoptosis-related protein expression was then analyzed using Western blotting. A: Western blotting of the band intensities of Bcl-xL, Bax, caspase-9, and caspase-3 after 24-h incubation with SN-38. B: SN-38 decreased the expression of Bcl-xL protein and increased the expression of phosphorylated caspase-9 and caspase-3 protein. Each bar indicates a mean ± S.E.M. The data are representative of 8 independent experiments. Details are described in Materials and Methods.

nuclear extracts of SN-38-treated cells using the EMSA. SN-38 increased the p53 DNA-binding activity

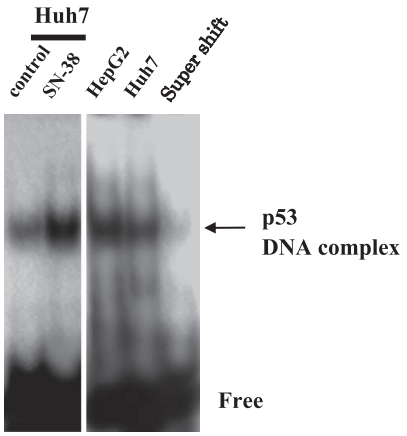


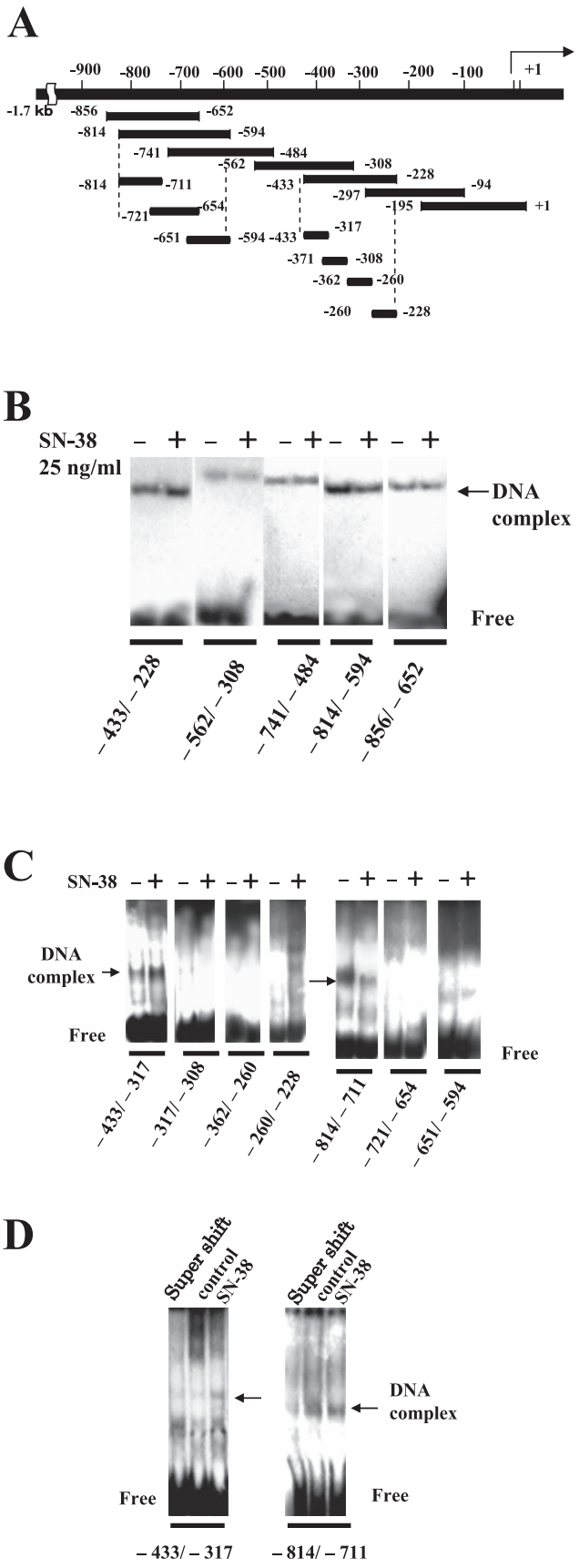
Fig. 5. DNA-binding of p53 in the nuclear protein of Huh7 cells after SN-38 treatment (lanes 1 and 2). Huh7 cells untreated with SN-38 (lane 1), Huh7 cells treated with SN-38 (lane 2). DNA-binding activity of p53 in Huh7 cells expressing mutated *p53* (a point mutation of codon220) was compared with wild-type p53 expressing HepG2 cells untreated with SN-38 (lanes 3 and 4). A100-fold excess of the unlabeled p53 ODN is added as competitor to show the specificity of the DNA-protein complexes formed in a sample of Huh7 cells (lane 5). The results are representative of 3 independent experiments.

compared with the controls (Fig. 5, lanes 1 and 2). We verified that Huh7 cells expressing mutated *p53* (a point mutation of codon 220) showed no difference in p53 DNA-binding activity compared with wild-type p53 expressing HepG2 cells (Fig. 5, lanes 3 and 4). Furthermore, the DNA-binding complex was completely inhibited by preincubation with a 100-fold excess of specific cold ODN (Fig. 5, lane 5).

Identification of DNA-binding sites of the p53 proximal promoter region for response to SN-38 in Huh7 cells

It is important to clarify whether SN-38 directly binds to the *p53* promoter upstream region to exert to the positive effects on *p53* gene transcription. To identify the SN-38-binding sites, we performed EMSA to determine whether SN-38 binds to the proximal promoter region (bases −856 to +1) using probes designed for approximately 200-bp promoter fragments from the 1.7-kb promoter region of the *p53* gene (Fig. 6A).

The DNA-binding activity to bases −433 to −228 *p53* promoter region of the nuclear proteins from SN-38-treated cells was increased compared with that in the untreated cells (Fig. 6B). Furthermore, DNA-binding activity to this region (bases −814 to −594) was down-regulated by SN-38 treatment (Fig. 6B). DNA-binding activity to bases −297 to −94 and the −195 to +1 region was not affected by SN-38 treatment (data not shown). These results suggest that promoter regions −433 to −228 and −814 to −594 are important for SN-38 binding.



We performed the EMSA using the approximately 100-bp oligo DNA containing bases −433 to −228 and −814 to −594, respectively (Fig. 6A). An increase in DNA-binding activity to bases −433 to −317 and inhibition of that to bases −814 to −711 was seen in SN-38-treated cells (Fig. 6C). Competition with a 100-fold excess of unlabeled double-stranded p53 promoter fragment oligo DNA specifically inhibited the binding of the complex (Fig. 6D).

Discussion

Irinotecan and its active metabolite SN-38 are both topoisomerase (Topo) I inhibitors. This drug is used in the cancer chemotherapy from the effectiveness in inhibiting the cancer cell proliferation, leading to the cell death (9, 12 – 14). The mechanisms of irinotecan in inhibiting cell differentiation or cell cycle arrest have been studied in various kinds of cancers, except HCC (25, 26). Thus we conducted present study. The human hepatocellular cell line Huh7 that we used was derived from Japanese patients with HCC, producing high levels alpha-fetoprotein and having a point mutation of *p53* (codon 220) as its feature. Although Huh7 cells have a *p53* mutation, this cell line has been shown to produce functional p53 protein in this study.

In the present study, we demonstrated that there are an increased number of TUNEL-positive cells and decreased cell proliferation in Huh7 cells, suggesting that apoptosis due to SN-38 treatment (Fig. 1). We subsequently attempted to clarify the effective SN-38 concentration for this process and demonstrated that incubation of Huh7 cells with SN-38 25 ng/ml increased apoptosis significantly by 7-fold at 24 h and decreased cell proliferation (Fig. 1). As indicated before, Huh7 cells have a *p53* mutation, which may suggest this cell line may not produce fully functional p53 protein, resulting different action in apoptosis and other cell functions. However, our results showed cell apoptosis, just as we also demonstrated the same phenomenon of apoptosis using HepG2 cells treated with SN-38. This

Fig. 6. Identification of the SN-38-binding sites of the p53 proximal promoter region. A: Schematic representation of the 1.7-kb promoter and designated *p53* promoter fragment oligo DNAs. B: Seven 200-bp fragments of *p53* promoter oligo DNA used in EMSA. Nuclear extract of SN-38-treated Huh7 cells was incubated for 24 h with the ^{32}P-labeled probe. Details are described in Materials and Methods. The results are representative of 3 independent experiments. C: EMSA was performed using ^{32}P-labeled bases −433 to −228 and −814 to −711 incubated with nuclear extracts in SN-38 treated Huh7 cells. D: A 100-fold excess of the unlabeled −433 to −317 and −814 to −711 fragments were also used as a competitor. The results are representative of 3 independent experiments.

would have meant that the p53 protein produced by Huh7 cells fully functioned in the same way as wild-type p53 protein from the HepG2 cells.

Generally, the apoptosis gene is an important factor in the regulation of cell death (10). The p53 tumor suppressor protein plays a key role in coordinating cell cycle arrest, DNA repair, and cell death after DNA damage (10, 27). We found that SN-38 (25 ng/ml) increased p53 phosphorylation at Ser^{15} and its protein in the nucleus of Huh7 cells (Fig. 2: A and B). It is reported that other anticancer drugs such as methotrexate trigger the nuclear accumulation of p53 protein (28, 29). Activation of *p53* involves posttranslational modifications of the p53 protein at multiple sites by phosphorylation. Multiple serine and three threonine residues have been reported to undergo phosphorylation in tumor suppression in response to cellular stresses (30, 31). Ser^{15} phosphorylation of p53 also has been shown to expose the Topo I inhibitor camptothecin and other chemotherapeutic agents in cancer cells. Recent studies have implicated that downregulation of *p53* gene activity and Ser^{15} phosphorylation may contribute to pathogenesis of liver cancer (23, 24). Therefore, we focused on the type of kinase that might contribute to the phosphorylation of p53 at Ser^{15} in SN-38-treated Huh7 cells and found that SN-38 not only induced accumulation of p53 protein but also phosphorylated p53 at Ser^{15} (Fig. 2). These results suggest that the SN-38-induced p53 increase may play an important role in the anticancer effects of SN-38 in HCC.

Additionally, to confirm the significance of increased p53 expression in SN-38-induced apoptosis, we evaluated the effects of pretreatment with *p53* AS ODN followed by SN-38. Pretreatment with *p53* AS ODN significantly decreased p53 protein expression and p53 phosphorylation at Ser^{15} in the nucleus of Huh7 cells (Fig. 2). *p53* AS ODN also significantly decreased the number of TUNEL-positive cells and amount of DNA fragmentation (Fig. 3: A and B). These findings led to the conclusion that pretreatment with *p53* AS ODN significantly decreases apoptosis and increases cell proliferation of SN-38-treated Huh7 cells. We speculated that SN-38-induced apoptosis was caused by DNA damage due to increased p53 expression in the cancer cells (32, 33). Results of this study support that the apoptosis due to SN-38 is mediated through p53 increases and activation in HCC cells.

The relative ratio of proapoptotic to antiapoptotic factors in the Bcl-2 family is believed to determine the threshold for activation of mitochondria-dependent apoptosis (34, 35). Accumulation of the proapoptotic protein Bax was induced to a greater degree in wild-type p53 than in p53-null colorectal cancer cells. Bax expression is under the transcriptional control of p53 (36, 37), and Bax can induce mitochondria-mediated apoptosis (38, 39). On the other hand, Hayward et al. reported that down-regulation of Bcl-xL can enhance the mitochondria-mediated apoptotic responses to SN-38 in colorectal cancer cells (40). Bcl-xL, but not Bcl-2, is highly expressed in human livers with HCC, and endogenously expressed Bcl-xL is important for the inhibition of apoptosis initiated by various cellular stresses in HCC-derived cell lines (41). Bcl-xL suppression also may be dependent on p53 activation. Our studies have shown that the expression of Bax increased and that of Bcl-xL decreased in SN-38-treated Huh7 cells accompanied by an increase in p53 expression (Fig. 4). As a result, *p53* AS ODN reverses the changes in expression of Bax and Bcl-xL in SN-38-treated cells, indicating that SN-38 induces the mitochondria-mediated apoptosis with the increase in p53 expression.

The caspase family plays an important role in downstream mechanisms of apoptosis (34, 42). The p53-induced apoptosis is also regulated in part by transcriptional activation of its target genes, and this process is dependent on the Apaf-1/caspase-9 activation pathway (43). Activation of caspase-3 represents a pivotal point in the apoptosis mechanism, and caspase-9, an activator of caspase-8, also accelerates apoptosis (42). In the present study, we clarified that SN-38 increases the expression of caspase-3 and caspase-9 in Huh7 cells (Fig. 4) and that these enzymes may be related to the apoptosis mechanism via p53 activation. Several studies have reported that loss of functional p53 reduces cellular sensitivity to chemotherapy agents (29, 32). In this study, we evaluated transcriptional targets of *p53* for the response to SN-38 as well as p53-dependent apoptotic effects. SN-38 increased p53 DNA-binding activity in Huh7 cells, indicating that p53 is a nuclear phosphoprotein that can bind to DNA. The p53 DNA-binding activity induced by SN-38 treatment in Huh7 cells appeared to be effective compared with wild-type p53-expressing HepG2 cells, suggesting that SN-38 directly binds to the p53 promoter region in both the cell lines.

In addition, it is important to clarify whether SN-38 induces binding to the *p53* gene promoter upstream region to exert its positive effects on *p53* gene transcription. Our studies showed the DNA-binding activity to the bases −433 to −317 of the *p53* promoter region of the nuclear proteins from SN-38-treated cells increased compared with that in untreated cells. On the other hand, DNA-binding activity to this region (bases −814 to −711) was down-regulated after incubation with SN-38 (Fig. 6).

Sequences similar to this DNA-binding motif were

found within two regions of the *p53* promoter. Sequence analysis (Gene Bank, http://www.genome.jp) indicated a putative GATA family site within the regions from base −433 to −317 of the p53 promoter and an NF-κB site within the regions from base −814 to −711. SN-38 may thus up-regulate the *p53* gene, which binds to the GATA site in the *p53* promoter region.

Gualdi et al. reported the GATA family transcription factor is a regulator of hepatic differentiation. GATA may also have exhibit inhibitory effects on down-regulation of a cancer gene (44). SN-38 blocks NF-κB activation and inhibits the growth responses of cancer cells (45). We hypothesize that SN-38 increases apoptosis during *p53* promoter activation with the regulation of these transcription factors in Huh7 cells. SN-38 may have anti-cancer effects by inducing apoptosis via p53 activation and by suppressing transcription factors for tumor regulation factors such as GATA or NF-κB in HCC.

In conclusion, our data suggest that the antitumor effects of SN-38 might include the mechanism of the mitochondria-apoptotic pathway inducing p53 activation. This newly discovered mechanism of action of irinotecan in chemotherapy might be useful in the treatment of patients with HCC.

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