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5-FU-Induced Apoptosis Correlates with Efficacy Against Human Gastric and Colon Cancer Xenografts in Nude Mice

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Abstract. *Apoptosis may be an important mechanism by which cancer cells are killed by certain agents. It is reported here that apoptosis is a key event in the killing of human tumor cells by 5-fluorouracil (5-FU) in vivo. Apoptosis induced by 5-FU was determined using two human gastrointestinal tumor xenografts serially transplanted into nude mice: a gastric carcinoma (SC-1-NU) highly sensitive to 5-FU and a colon carcinoma (Co-4) less sensitive to 5-FU. Apoptosis was assayed using the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling method in paraffin-embedded tissue sections, and by flow-cytometric analysis. Apoptosis-positive cells increased gradually during treatment. 24 hours after the inititation of 5-FU treatment a maximum, of 15.4 % of the Co-4 cells were apoptotic. 48 hours after the initiation of 5-FU treatment, apoptosis was found in 34% of the tumor cells in the SC-1-NU strain. Flow-cytometry demonstrated the increase of S-phase fractions in both strains after the administration of 5-FU, and this coincided with the appearance of apoptotic-positive cells. Although the intrinsic TS activities of two strains differed, TS activities were markedly suppressed in both strains immediately after the administration of 5-FU. Concentration of 5-FU in RNA (F-RNA) increased gradually in both strains, reaching a maximum 24 hours after the administration of 5-FU. These results suggest that apoptosis and inhibition of DNA synthesis induced by 5-FU are closely associated with its antitumor effect.*

Apoptosis, or programmed cell death, has recently been found to be an important regulator of various events, including developmental processes and clonal selection in the immune system(1,2). Radiation and chemotherapy have been shown to cause cell death by apoptosis (3,4,5,6). 5-FU and its derivatives are widely used in the chemotherapy of gastrointestinal cancer (7). The mode of action of this drug in relation to apoptosis is not well understood. Two main mechanisms of its antitumor effect have been proposed (8~13): a) 5-FU is metabolized to FdUMP, which suppresses TS and subsequently inhibits DNA synthesis, and b) FUTP is incorporated into cellular RNA, resulting in RNA distortion. In the present study, we investigated apoptosis induced by 5-FU and its relation to biochemical changes of the human tumor xenograft-nude mouse system. This report demonstrates that apoptosis induction is a mean by which 5-FU can kill human colon and stomach cancer cells growing as xenografts in nude mice.

Materials and Methods

Human tumor xenograft-nude mouse system. Experimental 5-FU chemotherapy was investigated using human tumor xenografts in nude mice using previously reported methods (14,15). In brief, BALB/c male nude mice (purchased from CLEA Japan Inc.) were maintained under specific pathogen-free conditions in the experimental animal center at the Tochigi Cancer Center. They were given sterile food and water *ad libitum.*

Abreviations: 5-FU, 5-fluorouracil; FdUMP, 5-fluorodeoxyuridine monophosphate; TS, thymidylate synthetase; FUTP, fluorouridine triphosphate; F-RNA, concentration of 5-FU in RNA; TdT, terminal deoxynucleotidyl transferase; dUTP, deoxyuridine triphosphate; dUMP, deoxyuridine monophosphate; dTMP, thymidine monophosphate.

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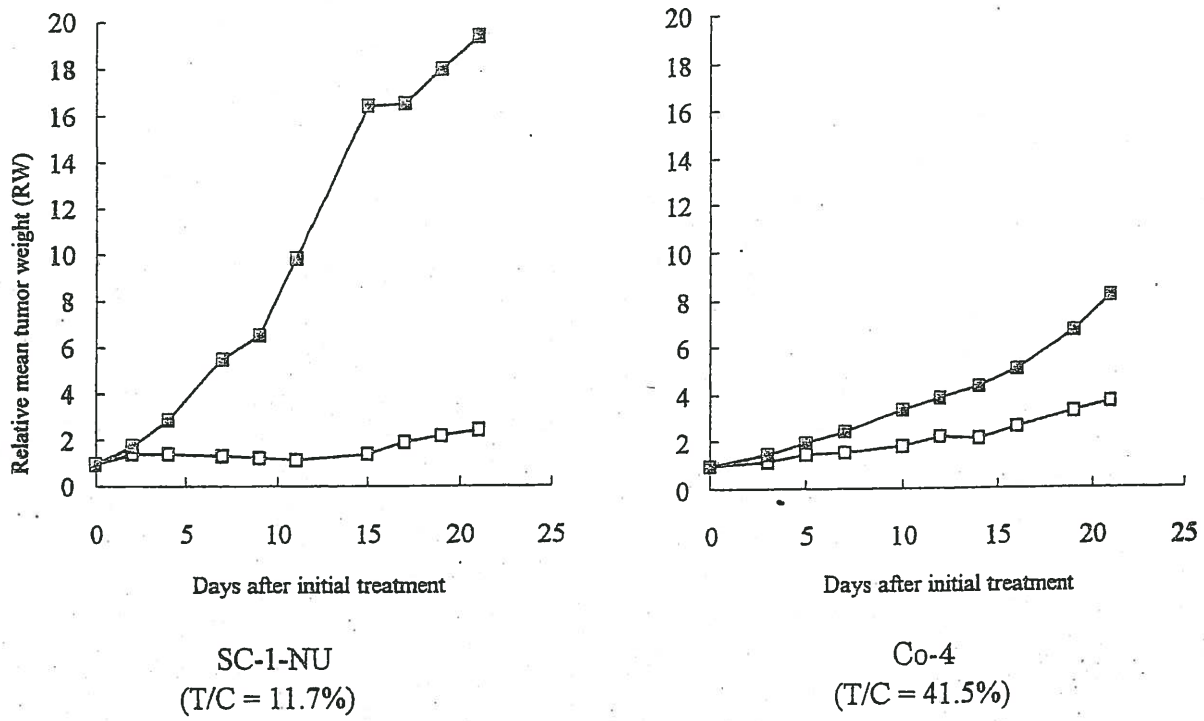

Figure 1. *Effects of 5-FU against two tumor strains.* -■-: *control,* -□-: *5-FU.*

Experiments were performed when the mice were 6-8 weelcs old and weighed 20-22g. The human gastric carcinoma xenograft SC- 1 -NU, a poorly differentiated adenocarcinoma established at the 2nd Department of Surgery, Nagoya University, and the human colon carcinoma xenograft Co-4, a poorly differentiated adenocarcinoma established at the National Cancer Center Research Institute, Tokyo, were used in this study (14). A single tumor fragment measuring 3 × 3 × 3 mm was inoculated into the subcutaneous tissue of the dorsum of ether-anesthetized nude mice using a trocar needle. The length and width of the tumors were measured with sliding calipers three times weekly by the same observer. The tumor weight was calculated according to the method of Geran *et al* (16) from linear measurements using the formula: tumor weight (mg) = length (mm) x [width (mm)]^2/2. When the tumor reached 100-300 mg, the tumor-bearing mice were allocated randomly to test groups each consisting of five or six mice.

Commercially available 5-FU was purchased from Kyowa Hakko Kogyo, Co. Ltd., Tokyo. 5-FU (60 mg/kg) was administered intraperitoneally (ip) in a schedule, of q4d × 3 determined in a previous study. This dose and schedule was the maximum tolerable drug load producing maximum antitumor activity (17). The relative mean tumor weight (RW) was calculated as RW =W_i / W_0, where W_i was the mean tumor weight at any given time and W_0 was the mean tumor weight at the time of initial treatment. The antitumor effects of the drug were evaluated in terms of the lowest T / C value (%) during the experiment, where T was the relative mean tumor weight of the treated group and C was the relative mean tumor weight of the control group at a given time.

For the determination of thymidylate synthetase (TS) activity, concentration of 5-FU in RNA (F-RNA), apoptosis as well as for flow-cytometric analysis, tumor-bearing nude mice were sacrified at 1, 2, 4, 6, 12, 24, 48 and 72 hours after the administration of 5-FU.

Assay. Apoptosis was assayed by the TdT-mediated dUTP-biotin nick end labeling method (18) using paraffin-embedded tissue sections, and flow-cytometric analysis. Collected tumors were divided into two pieces. One piece was fixed in 100% ethanol and embedded in paraffin wax for staining of apoptosis while the other was mechanically dissociated, washed with phosphate-buffered saline, and fixed with 70% ethanol at -20°C for flow-cytometric analysis.

Staining of apoptosis in tissue sections. Thin sections were cut (3 μm), mounted on poly-L-lysine-coated glass slides, covered, and dried. Staining of apoptosis was performed using the MEBSTAIN Apoptosis kit (Medical Biological Laboratories Co., Ltd., Nagoya, Japan). Sections were routinely deparaffinized and incubated with 40 μg/ml proteinase K for 30 minutes, at room temperature and then washed and immersed in TdT buffer. TdT (3.0 unit/μl) 5μl and biotinylated dUTP (250 μM) 5μl were added to cover the sections, and incubated for 60 minutes 37°C. The reaction was terminated by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 minutes room temperature, and then rinsed with double distilled water. After incubation with blocking solution for 10 minutes, the sections were incubated with 100μl avidin-FITC for 30 minutes at 37°C and rinsed with PBS. Sections were counter-stained with propidium iodide.

Flow-cytometric analysis. Flow-cytometric analysis was performed using the MEBSTAIN Apoptosis kit. After incubation with 40μg/ml proteinase K for 30 minutes the nuclear suspensions were incubated with TdT buffer, and then incubated with TdT and biotinylated dUTP for 60 minutes at 37°C. After incubation with ribonuclease A (Sigma, St. Louis, MO), the suspensions were incubated with propidium iodide. The flow-cytometric measurement was performed using a FACScan flow-cytometer (Becton Dickinson, San Jose, CA). The apoptotic fraction and cell cyde analysis were calculated with the LYSYS II program (Becton Dickinson).

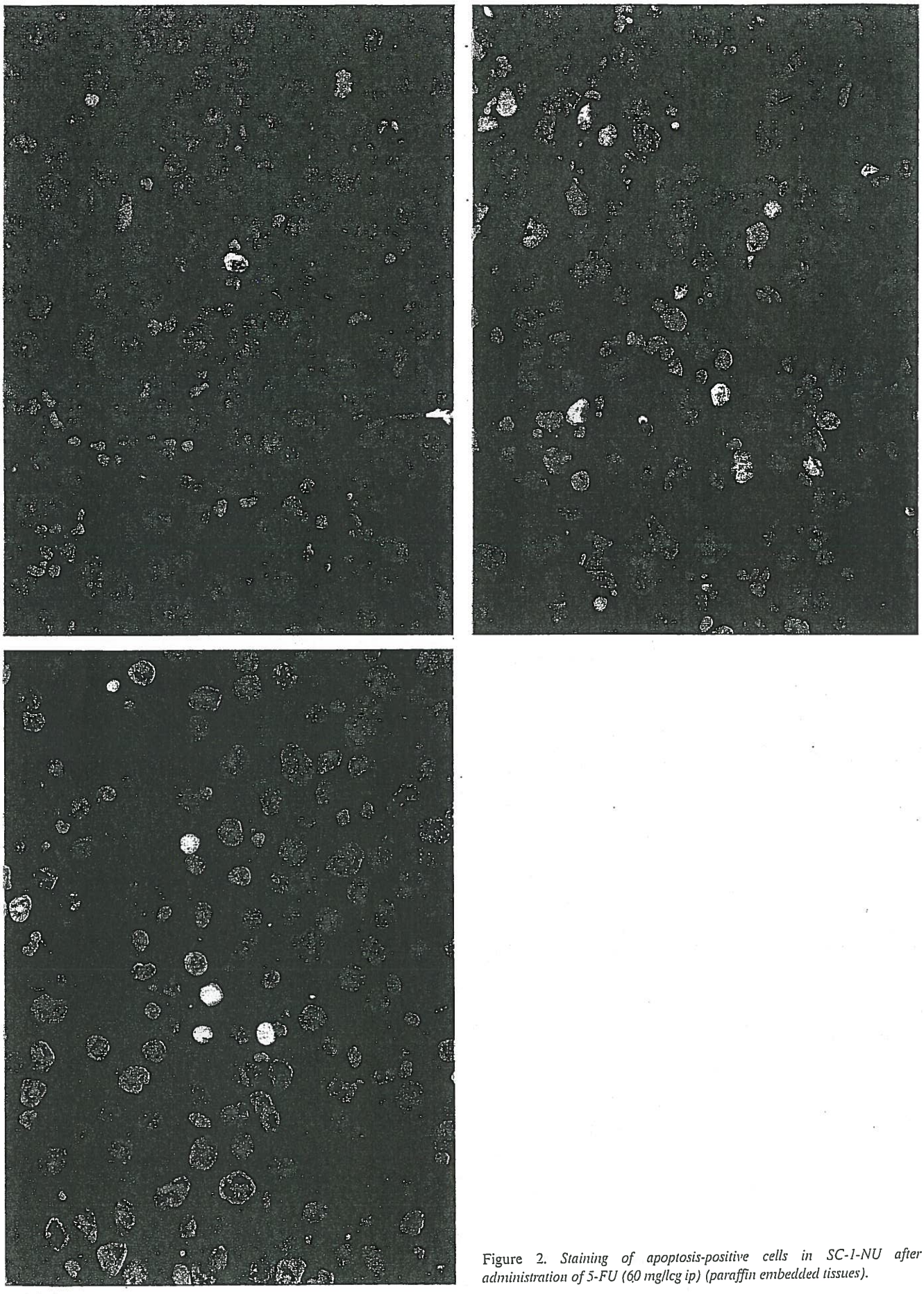

Figure 2. *Staining of apoptosis-positive cells in SC-1-NU after administration of 5-FU (60 mg/kg ip) (paraffin embedded tissues).*

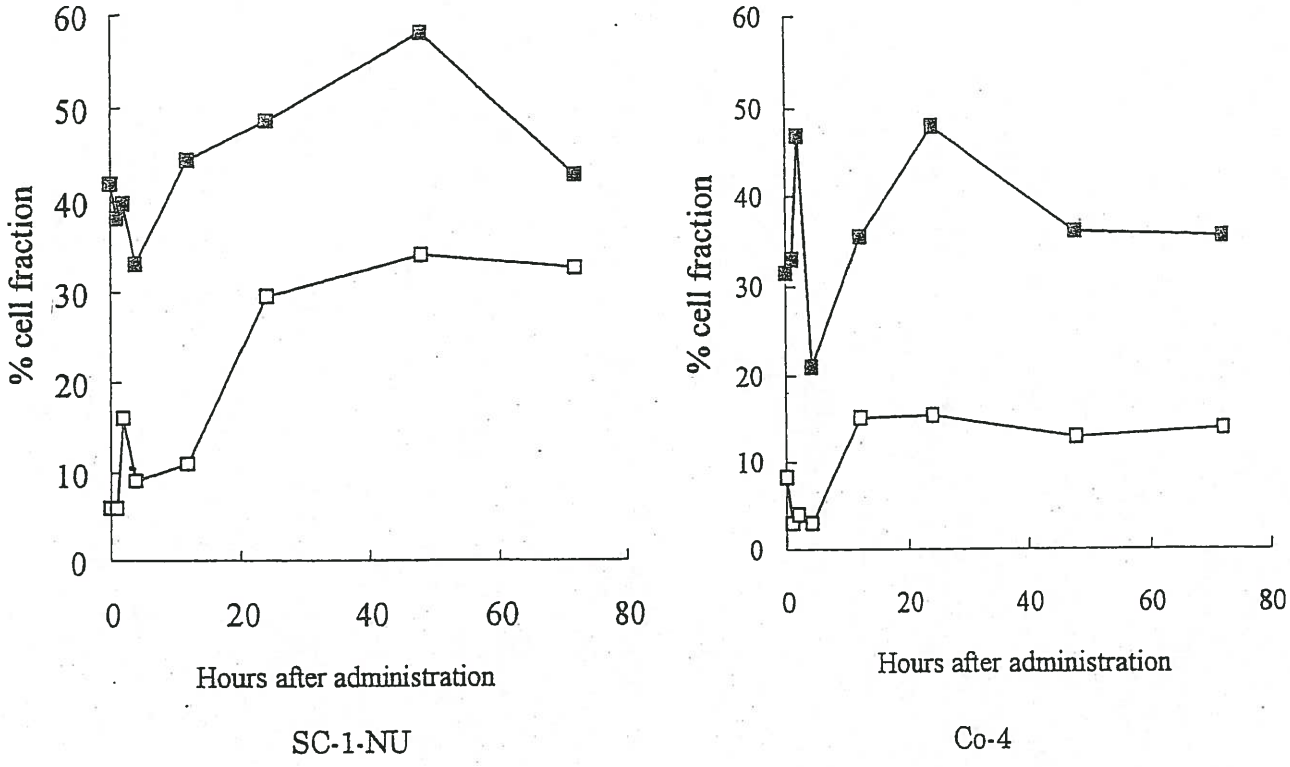

Figure 3. *Apoptosis-positive and S-phase cell fractions measured by flow cytometry.* -▦-: *S-phase,* -□-: *apoptosis.*

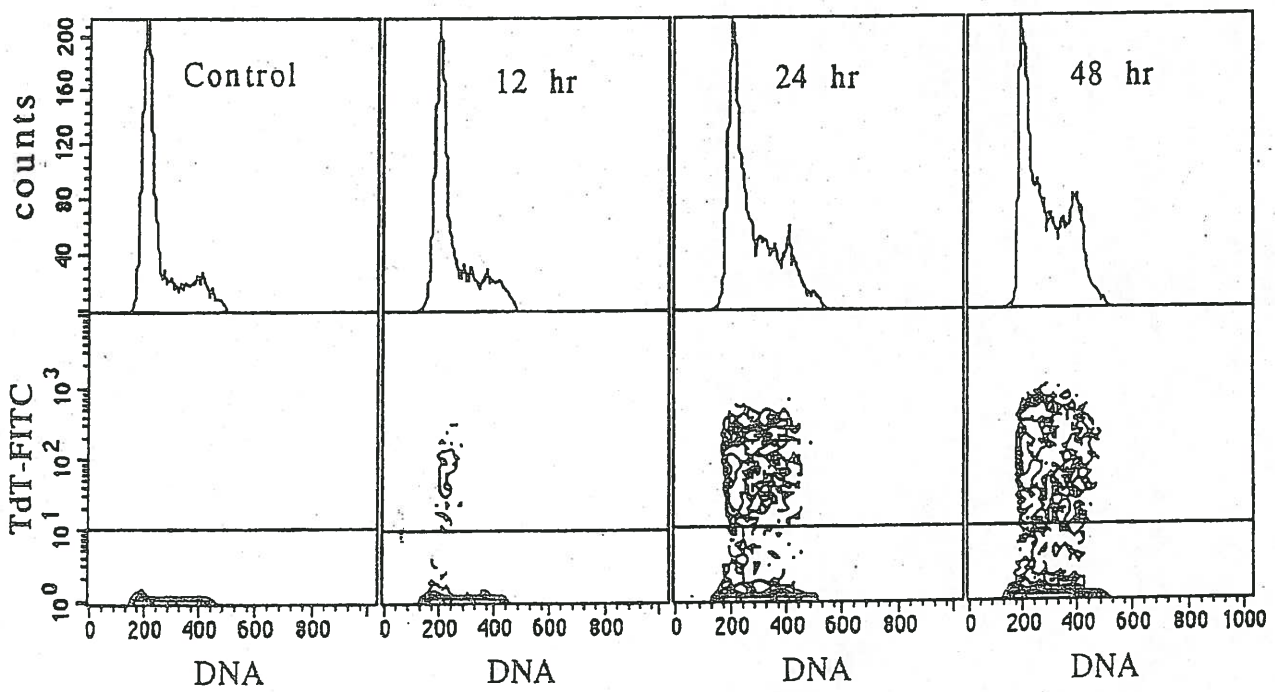

Figure 4. *Flow cytometric analysis of apoptosis and cell cycle distributions in the SC- 1 -NU tumor after the administration of 5-FU.*

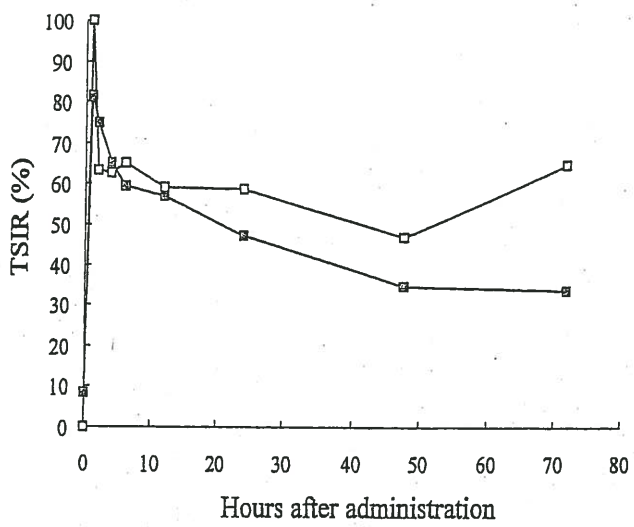

Figure 5. *Changes in thymidylate synthetase inhibition rate after administration of 5-FU.* -▩-: *SC-1-NU,* -□-: *Co-4*

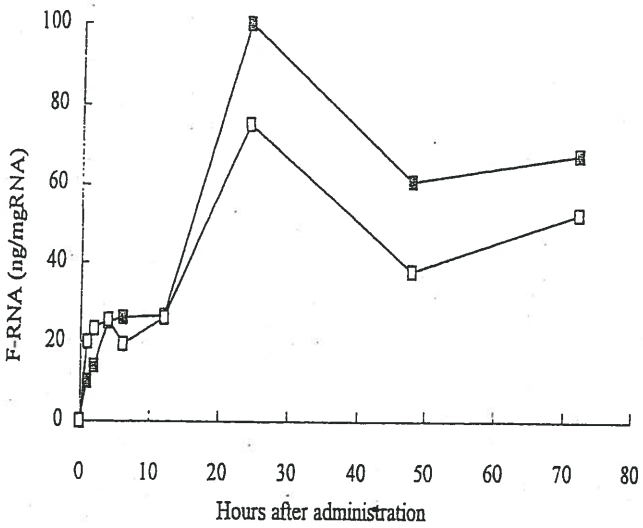

Figure 6. *Changes in F-RNA concentration after administration of 5-FU.* -▩-: *SC-1-NU,* -□-: *Co-4*

Assay of TS activity and F-RNA. The collected tumors were stored at -20°C before assay of TS and F-RNA. TS was assayed by a method modified from that of Spears *et al* (8). The TS inhibition rate (TSIR) was calculated as TSIR = (TStot - TSfree) / TS tot in percent, where TStot was the total TS and TSfree the free TS.

The concentration of F-RNA was assayed by gas chromatography-mass fragmentography (12,13).

Results

Efficacy of 5-FU on the growth of human tumor xenografts in nude mice. 5-FU showed potent efficacy against SC-1-NU compared with the control group with the lowest T/C value of 11. 7% (Figure 1 left). 5-FU also showed efficacy against Co-4. However, the lowest T/C value was 41.5% for Co-4, higher than that of SC-1-NU. Thus SC-1-NU and Co-4 were regarded as being sensitive and partially sensitive to 5-FU, respectively.

Apoptosis in 5-FU-treated tumors. Apoptosis in tissue sections of the 5-FU treated tumors was evaluated at 0, 1, 2, 4, 6, 12, 24, 48, and 72 hours after 5-FU administration. Figure 2 shows an example of the staining for apoptosis in the SC-1-NU tumor. Apoptosis-positive nuclei were seen in small numbers in controls with the numbers of apoptotic nuclei gradually increasing from 12 to 72 hours.

The apoptosis-positive fraction was also determined by flow-cytometry. Figure 3 demonstrates the apoptosis-positive fraction of the 5-FU-treated SC-1-NU tumor increased starting at 12 hours after 5-FU administration and reaching a maximum of 34 % by 48 hours.

The apoptotic-positive fraction of Co-4 also increased starting at 12 hours after 5-FU treatment and reaching a maximum of 15.4% by 24 hours after 5-FU administration. These results demonstrate that the maximum apoptosis-positive fraction after 5-FU treatment was larger in the 5-FU-sensitive SC-1-NU strain than in the partially-sensitive Co-4 strain.

The proportion of cells in the S-phase fraction increased after administration of 5-FU in both strains. The increase of S-phase cells coincided with the appearance of apoptosis-positive cells (Figure 3). The apoptosis-positive cell fraction appeared from early S-phase and increased throughout the S-phase in SC-1-NU (Figure 4). This was also observed in Co-4 (data not shown).

TS activity, and F-RNA in 5-FU treated tumors. The intrinsic TS activities of the two strains were 25.8 ± 5.7 pmol/g for SC-1-NU, and 5.86 ± 0.54 pmol/g for Co-4. Immediately after the administration of 5-FU, the inhibition of TS reached 86.0% in the SC-1-NU tumor and 100% in the Co-4 tumor by 1 hour and there after decreased biphasically (Figure 5).

The concentration of F-RNA increased gradually after the administration of 5-FU in both strains and reached 99.9 ng / mg RNA in the SC-1-NU tumor and 74.8 ng /mg RNA in the Co-4 tumor 24 hours after treatment (Figure 6).

Discussion

Although there are many reports of apoptosis-related cell death induced by anticancer agents in chemosensitive tumors (19-21), most however have been *in vitro.* In particular, the mechanism of apoptosis induced by 5-FU is much less well understood than apoptosis induced by topoisomerase inhibitors or cisplatin.

In this report, we investigated 5-FU induced apoptosis in human solid tumors *in vivo* growing in nude mice. In the

sensitive SC-1-NU strain, apoptotic cells occured in a maximum of 34 % of the tumor cells and in a maximum of 15.4 % of the cells of the less sensitive Co-4 strain. The results suggest that the extent of apoptosis could be associated with the antitumor effect of 5-FU.

Cell cycle analysis by flow-cytometry demonstrated the accumulation of tumor cells in the S-phase after 5-FU administration. The pattern of changes in the apoptosis-positive fractions was similar in both strains. Apoptosis-positive cells distributed from early to late S-phase in histograms of DNA content in both strains, This suggested that apoptosis induced by 5-FU occurred beginning in the early S-phase fraction in contrast to ordinary cell loss which occurs in the G_1 or G_0 -phase.

The antitumor mechanism of 5-FU is not clear. Two biochemical mechanisms involving the cytotoxic effects of 5-FU have been considered. One involves the conversion of 5-FU to FdUMP, which binds irreversibly to TS, leading to the inhibition of the conversion of dUMP to dTMP. This results in the depletion of dTMP and inhibition of DNA synthesis. The second mechanism relates to the conversion of 5-FU to FUTP, which is incorporated into RNA, resulting in RNA dysfunction. A recent study found that the main mechanism of action of 5-FU involves blocking of phosphorylation by inhibiting the activity of TS at doses equivalent to those used clinically (10,22).

In the present study, the intrinsic TS activity of the two strains studied was markedly different, though the TS activity was suppressed immediately and significantly after administration of 5-FU in both strains. The concentrations of F-RNA increased gradually after 5-FU administration and reached maximum levels 24 hours after administration in both strains.

Comparing these biochemical changes and the appearance of apoptosis as well as the cell cycle changes, apoptosis induced by 5-FU was observed after the inhibition of TS, correlating with the accumulation of tumor cells in the S-phase fraction caused by the inhibition of DNA synthesis. Yoshioka *et al* (23) reported that 5-fluorodeoxyuridine-induced apoptosis of FM3A cells *in vitro* was associated with a deoxyribonucleoside triphosphate pool imbalance. Their results were comparable with the present study in which 5-FU decreased dTMP, resulting in an imbalance of deoxyribonucleoside triphosphate associated with apoptosis of human solid tumors studied *in vivo*.Since the apoptosis-positive fraction of these tumors was correlated with their sensitivity to 5-FU, we suggest that apoptosis induced by 5-FU is closely related to its antitumor activity. Combination therapy with 5-FU to increase the apoptotic fraction may be a promising avenue of therapy for gastric and colon cancer.

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