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The p53 tumor suppressor gene in anticancer agent-induced apoptosis and chemosensitivity of human gastrointestinal cancer cell lines

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Abstract *Purpose*: While the target of many anticancer agents has been identified, the processes leading to killing of the cancer cells and the molecular basis of resistance to the drugs are not well understood. We used human gastrointestinal cancer cell lines and examined how anticancer agents induced cell killing and how the chemosensitivity of these lines was determined. *Methods*: Twelve gastrointestinal cancer cell lines were examined for the presence of either a wild-type or mutant p53 gene by direct sequencing. We also determined whether or not cell killing would occur when the cell lines were exposed to anticancer drugs. The sensitivity to the anticancer agents was determined based on colony formation. *Results*: All 12 gastrointestinal cancer cell lines carried either a wild-type or mutant p53 gene. Three lines, MKN45, MKN74 and COLO320, carried the wild-type p53 gene, and nine carried the mutant p53 gene. When three lines were exposed to the anticancer agents etoposide, doxorubicin (DXR) or 5-fluorouracil (5-FU), cell death ensued. In these cells, the population of cells in G_1 phase increased after exposure to high-dose anticancer agents, but cells in G_2 phase increased when exposed to low-dose anticancer agents. Our observations support the concept that cells carrying the wild-type p53 gene tend to be sensitive to etoposide and DXR and, in particular, deletion of the p53 function results in a greater resistance to anticancer agents. *Conclusion*: Based on our findings, human gastrointestinal cancer-related cell death apparently occurs via a p53-dependent pathway. A relationship was observed between the induction of cell death and chemosensitivity.

Key words Apoptosis · Gastrointestinal cancer · Cell cycle · Chemosensitivity

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Introduction

Apoptosis, or programmed cell death, has been given much attention and mechanisms whereby tumor cells can acquire or lose sensitivity to cytotoxic agents are being widely investigated. Apoptosis can be induced by cytotoxic compounds [1], growth factor deprivation [2], hormones [3], and growth arrest of cells overexpressing c-*myc* [4, 5] or p53 by blockers of G_1, S, or M phases of the cell cycle [6]. Typical events common to the action of most inducers of apoptosis include membrane and nuclear blebbing, DNA laddering [7–9] and activation of a Ca^{++}-dependent endonuclease [10].

The p53 tumor suppressor gene which has a role in some forms of apoptosis, seems to be the most commonly mutated gene in human cancer [11]. Products of the p53 tumor suppressor gene have been implicated as a molecule of central importance in this process [12], because of involvement in DNA damage-induced G_1 arrest [13], apoptosis [14], DNA repair [15], and gene amplification [16]. Wild-type p53 has been postulated to play a role in DNA repair, possibly by arresting cells in the late G_1 phase of the cell cycle, after irradiation [13]. Levels of p53 protein increase after treating cells with either ionizing radiation [17] or ultraviolet light [18]. Thus, the expression of mutant forms of p53 with altered cellular resistance to DNA damage can occur as a result of exposure to ionizing radiation [19].

The relationship between chemosensitivity and the presence of wild-type p53 or mutant p53 is unknown. Several studies have shown that p53 mutations reduce radiosensitivity [4, 20, 21] although this has not been

observed in all cases [22], possibly because of differences in the cell types studied and their susceptibility to apoptosis. The consequences of the p53 control system activation include a stable G_1 arrest and perhaps either enhanced DNA repair or a deletion of cells with DNA damage, via an apoptotic pathway.

In the present study, we examined the role of the p53 tumor suppressor gene in the cell cycle and cell death following exposure of human gastrointestinal cancer cells to etoposide, doxorubicin (DXR) and 5-fluorouracil (5-FU). We found that the status of the p53 gene was an important determinant of apoptosis in human gastrointestinal cancer cells.

Materials and methods

Cell culture

The cell lines used in the present study are listed in Table 1. Seven lines (MKN1, MKN28, MKN45, MKN74, KATOIII, SCH and AZ521) were derived from human gastric cancer, two (COLO201 and COLO320) were from human colon cancer, two (KSE-1 and T.Tn) were from human esophageal cancer, and one (RCM-3) was from human rectal cancer. All the cell lines, except KSE-1, were obtained from the Japanese Cancer Research Resources Bank Corporation (Tokyo, Japan). The KSE-1 cell line was established in our laboratory [23]. KATOIII, COLO201 and COLO320 cell lines were preserved in suspension culture, and nine lines were adherent cells. MKN1, MKN28, MKN45, MKN74, KATOIII, SCH, COLO201 and COLO320 cell lines were maintained as monolayers in RPMI-1640 (Gibco, Tokyo, Japan) with 10% fetal calf serum, AZ521 in MEM (Gibco, Tokyo, Japan) with 10% fetal calf serum, KSE-1 in Dulbecco's modified Eagle's medium (DMEM), T.Tn in 50% DMEM and 50% HAM (Nissui Pharmaceutical Co. Tokyo, Japan) with 10% fetal calf serum and RCM-3 in 50% RPMI-1640 and 50% HAM with 10% fetal calf serum.

The cell lines were cultured at 37 °C in an atmosphere comprising 95% air/5% CO_2. Exponentially growing cells were exposed to etoposide, DXR and 5-FU. Etoposide was obtained from Nihon Kayaku Co., (Tokyo, Japan) and DXR and 5-FU were obtained from kyowa Hakko Co., (Tokyo, Japan). Cells were exposed to etoposide, DXR and 5-FU in medium for 3 h, 3 h and 72 h, respectively. Cell survival following exposure to these anticancer agents was determined as follows. The cells (3×10^2) were plated in 60-mm dishes in the absence or presence of the drugs. The adherent cells were then washed three times with phosphate-buffered saline (PBS) and incubated at 37 °C in fresh medium, without the addition of any agent. KATOIII, COLO201 and COLO320 cell lines were suspended in 3 ml medium containing 0.1% Noble agar in closed tubes [24] after the cells were washed three times with PBS, and incubated at 37 °C in fresh medium, without any agent. The dishes were scanned using an inverted microscope 10–14 days later and colonies of more than 50 cells were counted. The control dishes contained about 200 colonies. The effects of treatment were then evaluated according to the rate of inhibition of colony formation.

RNA isolation, RT-PCR and sequencing

Total cellular RNA was isolated from monolayer cultures using the acid-guanidinium-thiocyanate-phenol-chloroform method [25]. The quality of RNA extracted was estimated on a 1% agarose gel stained with ethidium bromide, and the quantity of RNA was determined by $A_{260/280}$ measurements. RNA was immediately reverse transcribed to cDNA using the Perkin-Elmer system. RNA was added to a master mixture containing 1 µl RT buffer (2.5 U/ml), 1 µl dATP, dCTP, dGTP and dTTP, 2 µl $10 \times$ PCR buffer (100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 4 µl 25 m*M* $MgCl_2$, 1 µl 25 m*M* RNase inhibitor and 1 µl 2.5 m*M* oligo $d(T)^{16}$). The reaction was terminated by heating to 95 °C for 5 min.

The expression of p53 was determined by RT-PCR, the details of which have been described elsewhere [26]. This assay is based on amplification of the p53 gene of exons 4–9 and a β_2-microglobulin (β_2 m) which serves as an internal standard [27]. The used primers were as follows: $\beta_2 m$ (a) sense 5′-ACCCCCACTGAAAAAGATGA-3′, (b) antisense 5′-ATCTTCAAACCTCCATGATG-3′; *p53* (c) sense 5′-CATTCTGGGACAGCCAAGTCTGT-3′, (d) antisense 5′-CTGGGGAGAGGAGCTGGTGTTGT-3′.

PCR was done using cDNA in a final volume of 100 µl reaction mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) containing 2.5 units of thermostable DNA polymerase (AmpliTaq, Perkin-Elmer, Tokyo, Japan). The PCR cycle consisted of 2 min of denaturation at 94 °C, 2 min of primer annealing at 55 °C, and 2 min of extension/synthesis at 73 °C. The PCR primers were synthesized using a DNA synthesizer (Applied Biosystems, model 394). The PCR products were visualized by electrophoresis through a 1% agarose gel.

Direct sequencing of the PCR products was done using the dye terminator method (Applied Biosystems, Japan Co., Tokyo, Japan) and an ABI 373A DNA sequencer (Applied Biosystems) [28].

Analysis of the effects of anticancer agents on the cell cycle and apoptosis

For flow cytometry analysis, a monolayer of cells was rinsed twice with PBS. Cells were suspended at 5×10^5/ml in 0.2% (v/v) Triton-X-100 detergent (Katayama Chemical Co., Osaka, Japan).

Table 1 Relationship between the p53 gene status and apoptosis

Cells	Organ	p53 status	Sequence	Amino acid change	DNA ladder formation on exposure to anticancer agents
MKN45	Stomach	Wild-type			+
MKN74	Stomach	Wild-type			+
COLO320	Colon	Wild-type			+
MKN1	Stomach	Mutant	Codon 143 GTG to GCG	Val to Ala	−
MKN28	Stomach	Mutant	Codon 251 ATC to CTC	Ile to Leu	−
SCH	Stomach	Mutant	Codon 249 AGG to AGG AGG	Arg to Arg(Arg)	−
AZ521	Stomach	Mutant	Codon 303 AGC to AAC	Ser to Asn	−
COLO201	Colon	Mutant	Codon 198 GAA to GGA	Glu to Gly	−
KSE-1	Esophagus	Mutant	Codon 124 TGC to AGC	Cys to Ser	−
T,Tn	Esophagus	Mutant	Codon 268 GAA to GGA	Glu to Gly	−
KATOIII	Stomach	Deletion			−
RCM-3	Rectum	Deletion			−

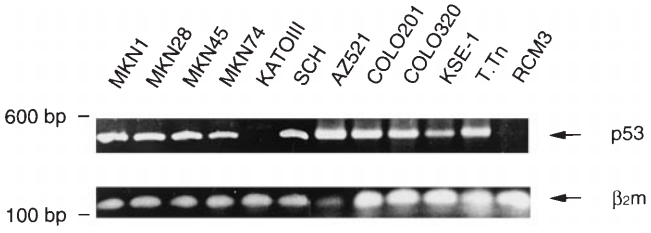

Fig. 1 Analysis of p53 cDNA in gastrointestinal cancer cells. The p53 cDNA was extracted using RT-PCR, as described in Materials and methods. The PCR products using β_2m and a p53 gene amplifier were 120 bp and 563 bp, respectively

Propidium iodide (PI, Sigma Chemical Co., St. Louis, Mo.) 100 µg/ml in sodium citrate buffer and 100 µl of 0.5 mg/ml ribonuclease A (RNAse, Sigma) were added to the cell suspension, and 2×10^4 cells were analyzed. The cell cycle distribution was analyzed using a FACScan (Becton Dickinson). Cell-FIT software was used for data collection and analysis. The gates were set on forward, and side-scattered light was used to eliminate debris and to select single cells. A total of 2×10^4 cells was used for cell cycle analysis of each sample.

An electrophoretic analysis of DNA fragmentation was performed on all the cell lines. After exposure to the anticancer drugs (etoposide, DXR, 5-FU), cells were collected, rinsed with PBS, lysed in 50 µl lysis buffer (10 m*M* EDTA, 50 m*M* Tris, pH 8.0, 0.1% sodium dodecyl sulfate, 0.5 mg/ml proteinase K), then incubated at 50 °C for 1 h. RNAase A (0.5 mg/ml) was added and the lysates were incubated for an additional 1 h. Two phenol extractions (equal volumes) were then carried out, followed by one chloroform extraction. DNA was precipitated with two volumes of iced ethanol and incubated at −80 °C for at least 2 h. DNA was pelleted by centrifugation at 13 000 rpm for 10 min at 4 °C. Pellets were air-dried for 30 min, resuspended in 50 µl Tris-EDTA, pH 8.0, and incubated overnight at 4 °C. DNA was electrophoresed in a 1.6% agarose gel in 0.5 × TBE running buffer (0.05 *M* Tris base, 0.05 *M* boric acid, 1 m*M* EDTA) for 2 h at 60 V.

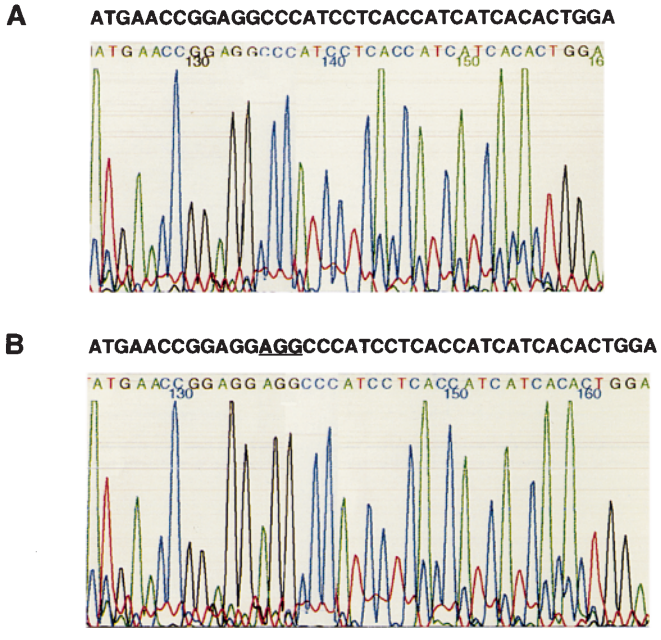

Fig. 2A,B Sequence analysis of the mutations. The sequences of p53 cDNA in MKN45 (**A**) and SCH (**B**) lines are shown. The p53 cDNA in MKN45 cell line was wild-type. The SCH cell line contained an insertion of AGG. The *underlining* indicates the position heterozygous for the extra AGG, with arginine as the amino-acid, resulting from the mutation in SCH cells

Results

p53 gene status in gastrointestinal cancer cell lines

Exons 4 to 9 of the p53 gene in twelve gastrointestinal cancer cell lines were amplified by RT-PCR and the sequence was examined, as described in Materials and methods. Ten cell lines were amplified by RT-PCR; the KATOIII and RCM-3 lines were not amplified (Fig. 1).

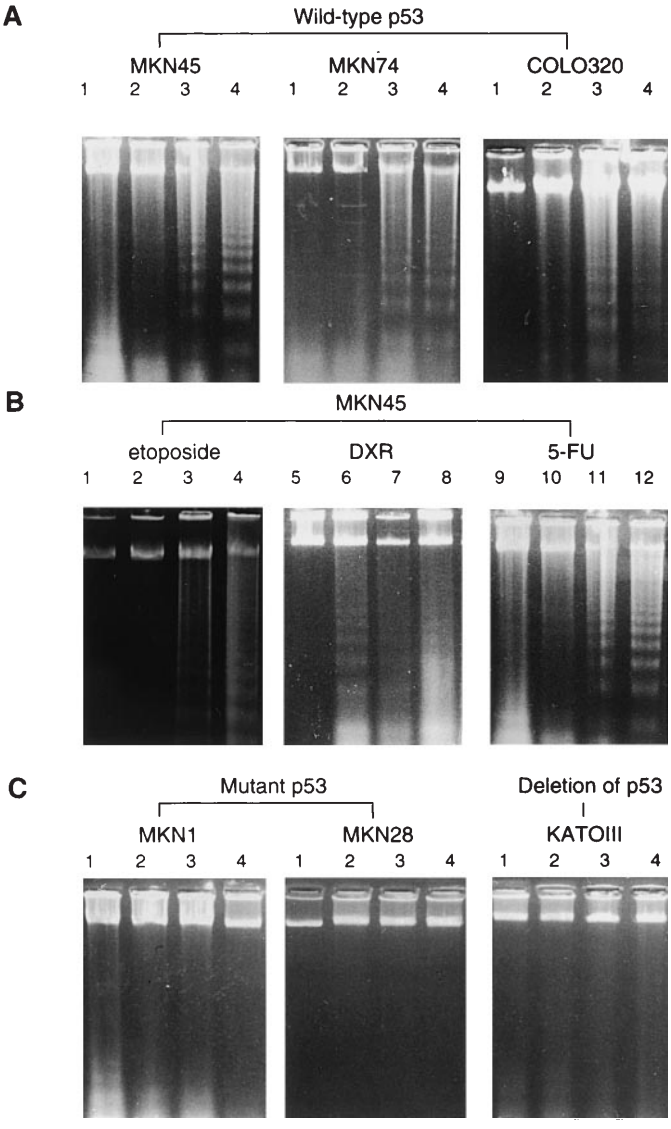

Fig. 3A–C Analysis of DNA ladder formation by cell lines exposed to etoposide, DXR and 5-FU. Each lane was loaded 10 µg DNA. **A**, **C** Each cell line was incubated with 0–500 µ*M* 5-FU for 72 h (*lane 1* no treatment, *lane 2* 100 µ*M* 5-FU, *lane 3* 200 µ*M* 5-FU, *lane 4* 500 µ*M* 5-FU). **B** MKN45 cells were incubated with 0–150 µ*M* etoposide for 3 h, or 0–150 µ*M* DXR for 3 h before the incubation for 24 h, and 0–500 µ*M* 5-FU for 72 h (*lane 1* no treatment, *lane 2* 50 µ*M* etoposide, *lane 3* 100 µ*M* etoposide, *lane 4* 150 µ*M* etoposide, *lane 5* no treatment, *lane 6* 50 µ*M* DXR, *lane 7* 100 µ*M* DXR, *lane 8* 150 µ*M* DXR, *lane 9* no treatment, *lane 10* 100 µ*M* 5-FU, *lane 11* 200 µ*M* 5-FU, *lane 12* 500 µ*M* 5-FU)

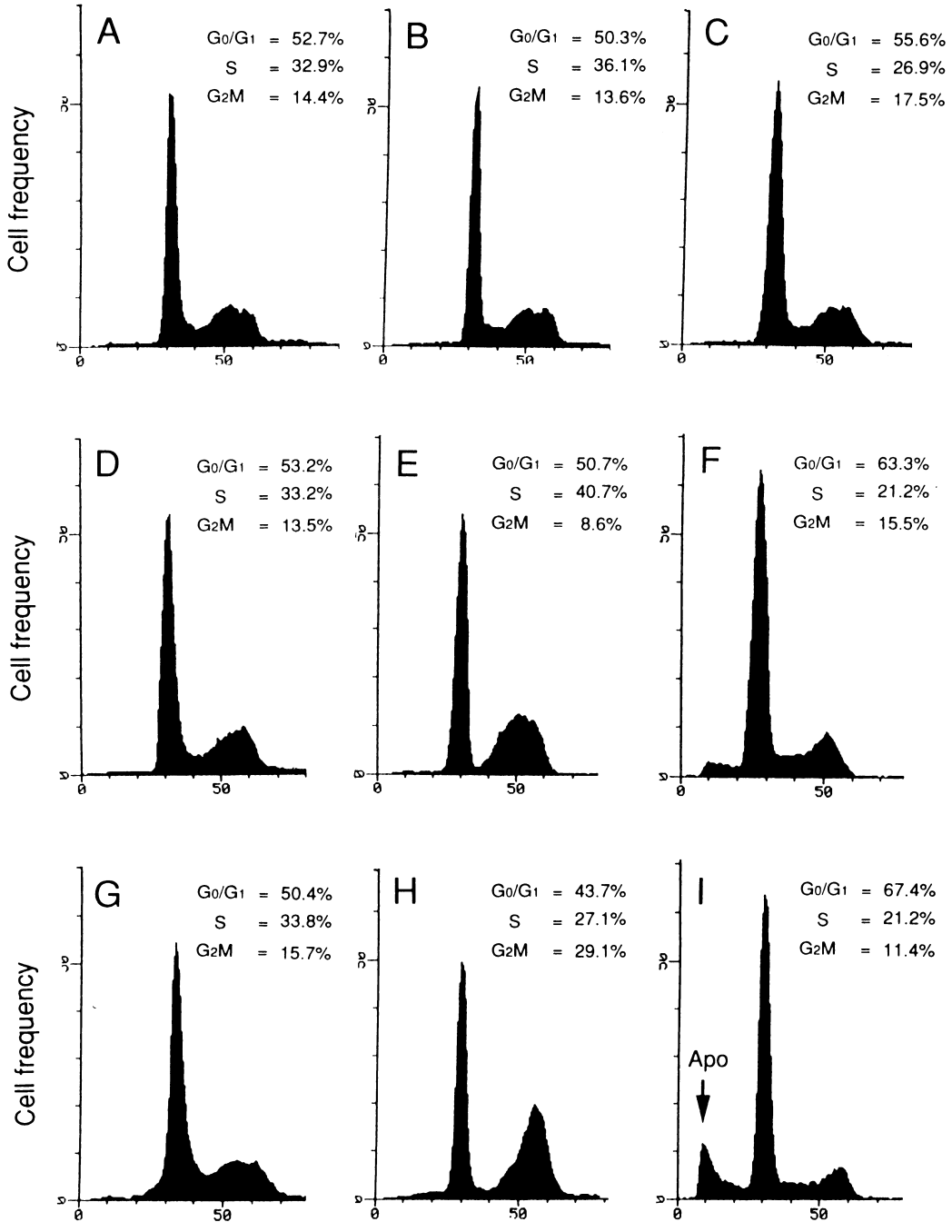

Fig. 4A–I The DNA content of a wild-type cell line. The cell cycle distribution of MKN45 cells after treatment with 0, 5 and 50 μ*M* DXR is shown. **A** No treatment, **B** 5 μ*M* DXR, **C** 50 μ*M* DXR, **D** culture for 12 h after no treatment, **E** culture for 12 h after exposure to 5 μ*M* DXR, **F** culture for 12 h after exposure to 50 μ*M* DXR, **G** culture for 24 h after no treatment, **H** culture for 24 h after exposure to 5 μ*M* DXR, **I** culture for 24 h after exposure to 50 μ*M* DXR. The apoptotic cells (Apc) are shown in Fig. 1.

These latter two lines lacked the p53 gene. Ten lines of p53 cDNA were sequenced using Sequenase and dye primers. Sequencing was performed using forward and reverse primers. We confirmed the mutation of p53 only when the same mutation was recognized by two primers, concomitantly.

MKN45, MKN74 and COLO320 cell lines carried the wild-type p53 gene (Fig. 2A) while mutation of the p53 gene was evident in the other seven cell lines. Six cell lines, but not the SCH cell line, had the point mutation, and the SCH cell line contained an insertion of AGG (Fig. 2B). The p53 gene status of the cell lines is given in Table 1.

Apoptosis induced by DNA damage

Each cell line was exposed to 0–150 μ*M* etoposide for 3 h or 0–150 μ*M* DXR for 3 h before incubation for 24 h, and 0–500 μ*M* 5-FU for 72 h. The MKN45, MKN74

and COLO320 cell lines showed DNA ladder formation. Figure 3A,B shows the DNA ladder formation in MKN45, MKN74 and COLO320 cell lines exposed to etoposide, DXR and 5-FU. In the MKN45 cell line, DNA ladder formation was seen when cells were exposed to more than 50 μ*M* etoposide, 10 μ*M* DXR and 100 μ*M* 5-FU (data not shown). None of the other nine cell lines which carried the mutation or had lost the p53 gene showed DNA ladder formation (Fig. 3C). DNA ladder formation in relation to p53 status is summarized in Table 1.

Cell cycle distribution

There were no significant changes in cell cycle distribution in the MKN45 cell line right after exposure to DXR, but at 24 h most cells were in the G_1 and G_2M phases. The cell cycle distribution in the control culture was 52.7% G_1, 32.9% S, and 14.4% G_2M phase (Fig. 4A). The cell population in the S-G_2M phase in culture at 12 h increased after exposure to 5 μ*M* DXR and this increase continued for 24 h (Fig. 4E,H). After exposure to 50 μ*M* DXR, apoptotic cells, which were smaller than cells in G_1 phase, were observed in culture at 12 h and the cells increased in the culture at 24 h (Fig. 4F,I). At 24 h after exposure to 50 μ*M* DXR, the distribution was 67.4% G_1, 21.2% S, and 11.4% G_2M phase (Fig. 4I), and cell populations in the G_1 phase also increased. Treatment with low-dose DXR thus induced cell accumulation in the G_2 phase and high-dose DXR induced accumulation in the G_1 phase.

Chemosensitivity of gastrointestinal cancer cell lines

The chemosensitivities of 11 gastrointestinal cancer cell lines, excluding the AZ521 cell line which did not form colonies, are shown in Fig. 5AB. The extent to which p53 status was a determinant of chemosensitivity was investigated, based on colony formation. The chemosensitivity to etoposide, DXR and 5-FU in these cell lines appeared to depend on the p53 gene status. The presence of the mutant p53 gene tended to inhibit cell survival compared with survival of cells with the wild-type p53 gene on exposure to etoposide and DXR (Fig. 5A). Cell lines KATOIII, COLO320, KSE-1 and RCM-3 were not chemosensitive to etoposide or DXR, in contrast to the other cell lines (Fig. 5B). COLO320 and KSE-1 lines showed overexpression of the MDR gene (data not shown) and in the KATOIII and RCM-3 cell lines, the p53 gene was deleted. Cancer cells possessing wild-type p53 in general tended to be more chemosensitive to etoposide and DXR (Fig. 5A). Cells

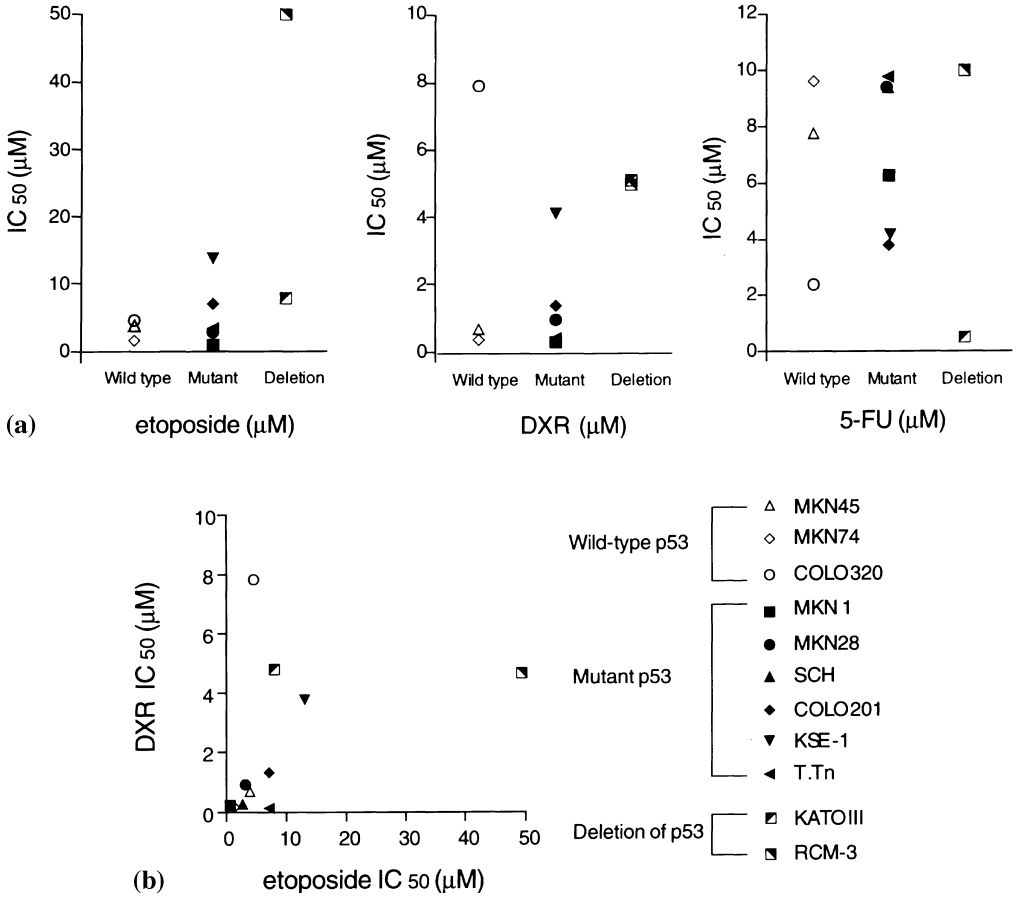

Fig. 5A,B **(A)** Apoptotic cells, which were smaller than cells in G_1 phase were observed. Relationship between p53 status and sensitivity to etoposide, DXR and 5-FU. Exponentially growing cells were treated with each drug as described in Materials and methods. Each point represents the mean IC_{50} from two experiments in each of which duplicate samples were counted. The IC_{50} values for each agent in three wild-type p53 cell lines, eight mutant p53 cell lines, and two cell lines with the p53 gene deleted are shown. COLO320 (○) and KSE-1 (▼) cell lines showed overexpression of the MDR gene. **(B)** Relationship between the IC^{A}_{50} value of etoposide and DXR for all 11 cell lines

with a deleted p53 gene tended to have reduced chemosensitivity, in comparison with cells with the wild-type or mutant p53 gene.

Discussion

We demonstrated a correlation between the status of the p53 gene and the apoptosis induced by DNA damage in human gastrointestinal cancer cell lines. In esophageal, gastric, colon and rectal cancer cells, which carried the wild-type p53 gene, apoptosis was induced by DNA damage. Yonish-Rouach et al. [29] were apparently the first to demonstrate this p53-related apoptosis. Clarke et al. and Lowe et al. [30, 31] suggested that while wild-type thymocytes undergo apoptosis, homozygous null p53 thymocytes are resistant to the induction of apoptosis by exposure to ionizing radiation or to anticancer drugs. This has also been noted in mouse embryonic fibroblasts [14] and erythroleukemia cells [6]. Similarly, we found that apoptosis induced in gastrointestinal cancer cells occurred via a p53-dependent pathway which was not correlated with the character of the cell line (e.g. in terms of differentiation or organ).

p53 functions as a transcription factor and thus triggers G_1-S and S-G_2M transitions. In many studies, the G_1-S transition has been detected but not the S-G_2M transition. It has also been shown that cells expressing wild-type p53 exhibit G_1 and G_2 arrest, while those overexpressing mutant p53 genes exhibit only G_2 arrest [32]. Our results indicate that cell populations in the G_1 and G_2 phases increased in the presence of anticancer drugs. In particular, high doses of drug increased the population in the G_1 phase, while low doses increased the population blocked at the G_2 phase. Apoptotic cells were detected after populations in the G_1 phase had increased (Fig. 4F,I), which means that cell populations in the G_1 phase had become apoptotic. The p53-dependent G_1 and G_2 accumulation was noted in the gastrointestinal cancer cell lines used in this study. We suggest that cells accumulating in the G_1 phase transferred p53-dependent apoptosis, because in the KATOIII cell line with the p53 gene deleted, anticancer agents increased populations in the G_2 phase (Data not shown).

A correlation between p53 status (wild-type versus mutant) and radiosensitivity or chemosensitivity in a variety of cells has been demonstrated [22, 33]. It has been reported that mutant p53 cells were more resistance to radiation than wild-type p53 cells [34, 35]. Our results indicate that chemosensitivity (to etoposide and DXR) of cells with wild-type p53 tended to be greater than that of cells with mutant p53, but this difference was not found for 5-FU. Inhibition of thymidylate synthase (TS) by 5-FU cytotoxixity has been reported to lead to DNA strand breakage [36]. However, it has also been reported that the major function of 5-FU is not the inhibition of TS, but the incorporation of 5-FU into RNA [37]. We did not examine the effects of exposure to 5-FU on TS, but we suggest that the differences in TS between the cell lines might be a result of differences in chemosensitivity to 5-FU. The deletion of p53 function enhanced cellular resistance to etoposide and DXR, but only two cell lines were examined. Also, the chemosensitivity of the SCH cell line containing the insert AGG showed almost no change compared to that of the wild-type cell lines. Our findings suggest that deletion of the p53 gene may be associated with decreased sensitivity to DNA-damaging agents in human gastrointestinal cancer cells. This decreased sensitivity may be due to avoidance of wild-type p53-mediated apoptosis. However, other factors modulating p53 function could also influence cellular resistance to anticancer agents, including $p21^{waf1/Cip1}$[21].

In summary, anticancer agents apparently induce apoptosis via a p53-dependent pathway in human gastrointestinal cancers. This has commonly been seen for the mutant p53 gene in human cancers [38]. In the clinical setting, the majority of gastrointestinal cancers are rarely responsive to both radiotherapy and chemotherapy, but recent observations do suggest a strong correlation between a tumor's p53 status and the response of the patient to chemotherapy and radiation [39, 40].

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