Jpn. J. Cancer Res. 88, 39–43, January 1997

Induction of p53-independent Apoptosis Associated with G2M Arrest Following DNA Damage in Human Colon Cancer Cell Lines

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The tumor suppressor p53 protein induces apoptosis in response to various kinds of DNA damage in normal cells, but it is still unclear whether or not apoptosis induced by DNA damage correlates with the p53 status in tumor cells. We determined the status of p53 by functional analysis of separated alleles in yeast in five human colon cancer cell lines, SW-480, SW-620, DLD-1, COLO320 and LS174T and investigated whether p53 is necessary for apoptosis and cell cycle arrest after treatment of the cells with a DNA-damaging agent, etoposide (VP-16), or γ-irradiation. Of these cell lines, only LS174T expresses a functional p53. Apoptosis was detected in SW-480 and COLO320 cell lines, but not in the other cell lines, including LS174T cell line with a normal p53 function. Furthermore, cell cycle analysis revealed accumulation in the G2M phase preceding induction of apoptosis in SW-480 and COLO320 cells, but not in the other cells. These results suggest that apoptotic induction by DNA damage is not necessarily related to p53 status and that induction of p53-independent apoptosis following DNA damage may correlate with G2M arrest in the cell cycle, at least in the colon cancer cell lines used in this study.

Key words: p53 — G2M arrest — Apoptosis — Human colon cancer

Apoptosis, as opposed to necrosis, is considered to be a programmed and active cell death that follows various stresses such as starvation, anticancer drugs and irradiation,[1] and is defined at present in terms of both morphological (cell shrinkage, chromatin condensation and presence of apoptotic bodies) and biological (internucleosomal DNA fragmentation) criteria.[2] It has been shown that genotoxic stress due to anticancer drugs such as the topoisomerase I (Topo I) and topoisomerase II (Topo II) inhibitors camptothecin and etoposide (VP-16), respectively, or ionizing irradiation, induces apoptosis.[3,4] However, details of the underlying mechanism subsequent to DNA damage have not been clarified as yet. Such damage is known to cause proliferating cells to arrest in the G1 or the G2 phase of the cell cycle, these G1 and G2 arrestpoints, referred to as cell cycle checkpoints, being evolutionally well-conserved between *Saccharomyces cerevisiae* and human.[5]

In mammalian cells, G1 arrest after DNA damage is achieved through the function of p53, which is commonly mutated in human tumor cells.[6] Accumulating evidence indicates that most tumor cells with *p53* mutations have an abrogated ability to be arrested in the G1 phase and are insensitive to induction of apoptosis after genotoxic stress.[7,8] However, several studies have demonstrated that there are some cell lines which express no functional p53 and nevertheless undergo apoptosis following treatment with high doses of ionizing radiation or anticancer drugs, suggesting that p53 is not the only mediator of apoptosis provoked by DNA damage.[9,10]

In the present study, we screened the p53 status of human colon cancer cell lines and investigated its relevance to the induction of apoptosis and cell cycle control after exposure of the cells to VP-16 or γ-radiation.

MATERIALS AND METHODS

Anticancer drug and cell lines VP-16, an anticancer drug which inhibits Topo II activity, was purchased from Sigma Chemical Co. (St. Louis, MO) and was used at the indicated concentrations after having been dissolved in dimethyl sulfoxide. LS174T, DLD-1 and COLO320 were obtained from the Japanese Cancer Research Resources Bank (JCRB) (Tokyo). SW-480 cells were a kind gift from Dr. Nishibe and SW-620 cells were purchased from Dainippon Seiyaku Co., Ltd. (Tokyo). These two cell lines were derived from the same patient, from the primary lesion (SW-480) and from a metastatic lymph node lesion (SW-620).[11]

p53 status The status of p53 in the five cell lines was determined by functional analysis of separated alleles in yeast (FASAY) as described previously.[12] This method evaluates whether *p53* mRNA expressed in the cells has the ability to trans-activate when reverse-transcribed and then expressed in yeast. Structural changes in the *p53* gene in the five colon cancer cell lines were previously examined by others.[13]

Cell culture and measurement of cell viability The cells were grown at 37°C in the presence of 5% CO_2 in

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Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (GIBCO BRL), 100 IU/ml penicillin G and 100 IU/ml streptomycin. Cell viability was assayed using the MTT assay (Cell Titer 96, Promega Co., Madison, WI)[14] after incubation in 96-well microplates with VP-16 for 72 h. The absorbance of the wells at 570 nm was automatically read using an ELISA Reader (Otsuka Electronics Co., Ltd., Osaka).

DNA fragmentation After VP-16 treatment or γ-irradiation, cells were washed twice with PBS, pelleted by centrifugation and resuspended in 300 μl of lysis buffer containing 500 m*M* Tris-HCl, pH 9.0, 0.2 m*M* EDTA, 10 m*M* NaCl, 1% SDS and 1mg/ml Proteinase K. After incubation at 48°C overnight, RNase A was added at a final concentration of 0.1 mg/ml at 37°C for 3 h. Then, cellular DNA was extracted by standard phenol/chloroform extraction.

The DNA was separated in 1.5% agarose gels and visualized by UV illumination after ethidium bromide staining.

Cell morphology Untreated and VP-16-treated cells were attached to glass slides using Cytospin, stained with May-Giemsa and observed under a microscope.

Cell cycle analysis The cells treated with VP-16 or γ-irradiation were stained with propidium iodide (PI) using Cycle Test Plus (Becton Dickinson Immunocytometry Systems, San Jose, CA) and examined in a FACSort™ (Becton Dickinson). Cell cycle distribution was analyzed with the LYSYS II software program (Becton Dickinson).

RESULTS

p53 status The FASAY showed that the functional p53 status in SW-480, SW-620, COLO320 and DLD-1 cell lines was mutant and that the LS174T cell line was functionally wild type. The results were consistent with the previous report, in which "structural" *p53* mutations were analyzed in the colorectal cancer cell lines (Table I).

Table I. Characteristics of 5 Human Colon Cancer Cell Lines

Cell line	IC_{50} (μM)	p53 status	G_2M arrest	Apoptosis
SW-480	3.0	mt	+	+
SW-620	0.7	mt	−	−
LS174T	10.9	WT	−	−
DLD-1	14.1	mt	−	−
COLO320	12.5	mt	+	+

mt, mutant; WT, wild type.

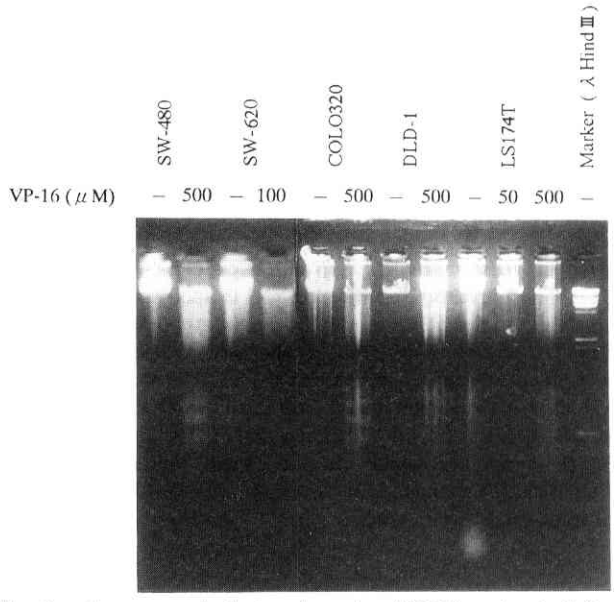

Fig. 1. Agarose gel electrophoresis of DNA extracted from SW-480, SW-620, COLO320, DLD-1 and LS174T cells following VP-16 treatment. These cells were exposed to VP-16 at the concentrations indicated for 60 h. Cellular DNA (1.5 μg/lane) was separated in 1.5% agarose gel and visualized by UV illumination after ethidium bromide staining.

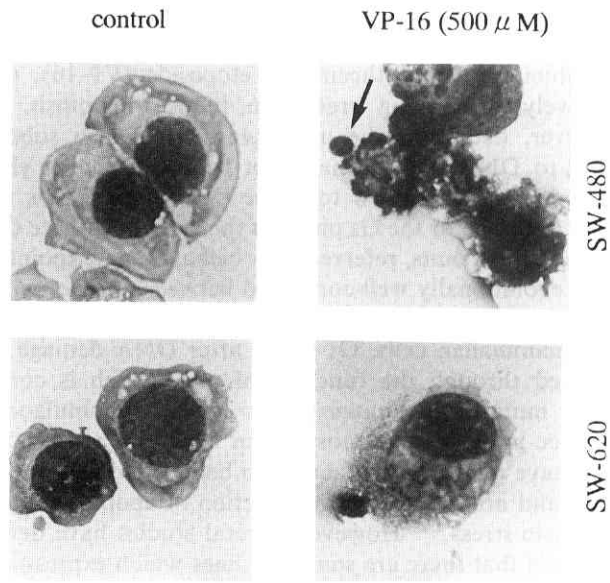

Fig. 2. Morphology of SW-480 and SW-620 cells treated with VP-16. The SW-480 and SW-620 cells were incubated for 60 h either alone or with VP-16 (500 μM). Cells attached to the slide glass were stained with May-Giemsa. The arrow shows an apoptotic body.

Cell viability after exposure to VP-16 in human colon cancer cell lines The SW-480, SW-620, COLO320, DLD-1 and LS174T cells (1×10^4) were plated in 96-well microculture plates. After incubation for 24 h, various concentrations of VP-16 were added for an additional 72 h. Percent cell viability was determined as percent ratio of absorbance of treated cells to that of control cells. The concentrations of VP-16 which gave 50% cell viability (IC_{50}) in SW-480, SW-620, COLO320, DLD-1 and LS174T cells were 3.0, 0.7, 12.5, 14.1 and 10.9 μM, respectively (Table I).

DNA fragmentation after VP-16 treatment in human colon cancer cells The five cell lines were incubated with the indicated concentrations of VP-16 for 60 h based on the IC_{50} values. VP-16 at concentrations of over 30 times the IC_{50} induced DNA fragmentation in SW-480 and COLO320 cells, but not in the other cells (Fig. 1). No DNA fragmentation was observed in any of the cell lines treated with the IC_{50} concentration (data not shown).

Cell morphology The SW-480 and SW-620 cells were incubated with VP-16 at the concentration at which SW-480 cells underwent DNA fragmentation. The SW-480 cells displayed morphological features of apoptosis such as cell shrinkage, nuclear condensation and the presence of apoptotic bodies. Cell swelling but no apoptosis-specific changes were observed in SW-620 cells (Fig. 2).

Cell cycle analysis after VP-16 treatment The SW-480, SW-620, COLO320, DLD-1 and LS174T cells were stained with PI after incubation with VP-16 at 500 μM for 24 h. The G2M fraction of the SW-480 and COLO320 cells increased to approximately 50% after VP-16 treatment. No obvious change in cell distribution was observed with the other cell lines (Fig. 3). These results, combined with the data described above, suggested that G2 arrest in the cell cycle is closely related to the induction of apoptosis following VP-16 treatment in these cell lines.

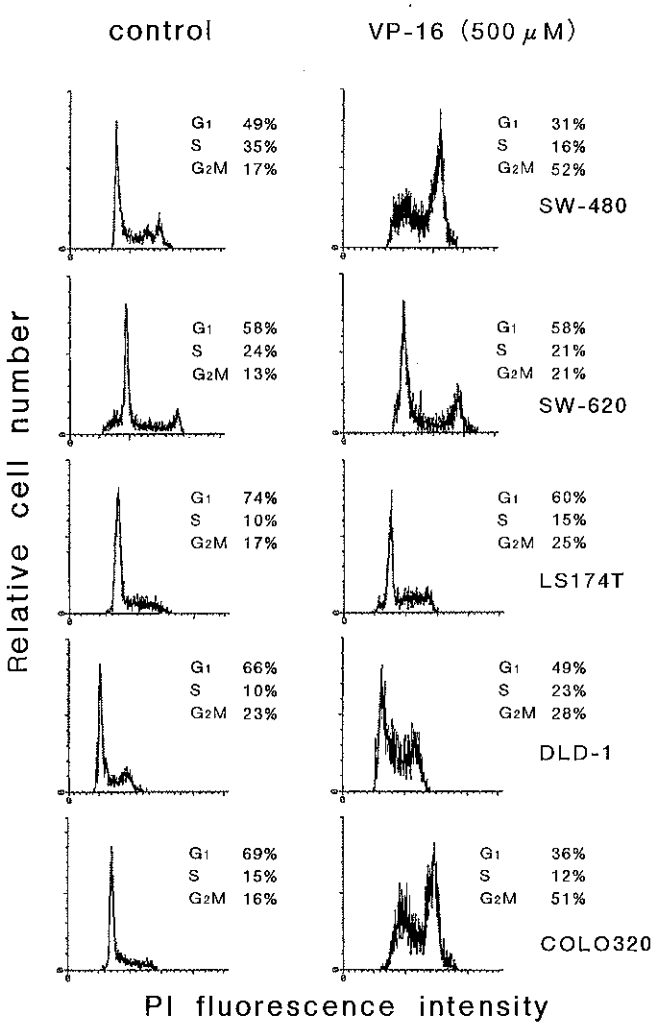

Fig. 3. Effects of VP-16 on the cell cycle in human colon cancer cell lines. SW-480, SW-620, LS174T, DLD-1 and COLO320 cells were incubated for 24 h either alone or with VP-16 (500 μM). The cell cycle distribution was analyzed using propidium iodide staining.

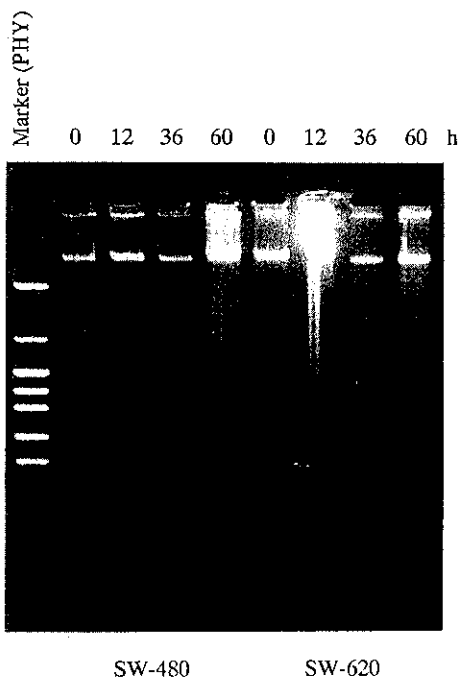

Fig. 4. Agarose gel electrophoresis of DNA extracted from SW-480 and SW-620 cells following γ-irradiation. Cellular DNA was prepared at the times indicated (h) after γ-irradiation (20 Gy) and after treatment as described in the legend to Fig. 1.

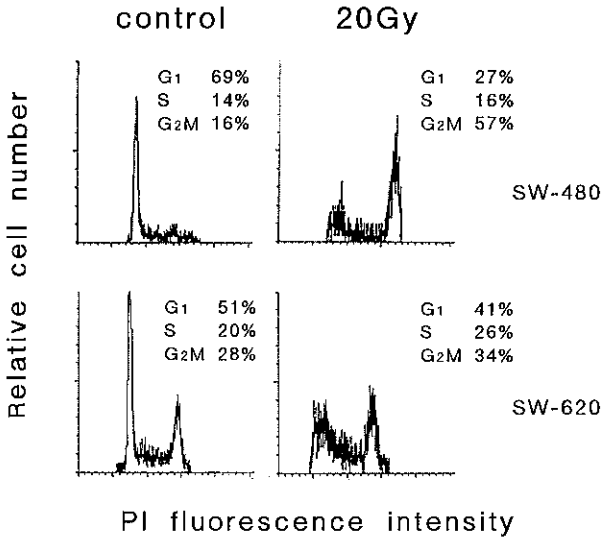

Fig. 5. Effects of γ-irradiation on the cell cycle in SW-480 and SW-620 cells. The SW-480 and SW-620 cells were incubated for 48 h either alone or after γ-irradiation (20 Gy).

DNA fragmentation and cell cycle analysis after γ-irradiation To investigate whether γ-irradiation, another genotoxic stress, can also induce apoptosis involving G2 arrest, SW-480 and SW-620 cells were irradiated at the dose of 20 Gy and their cell cycle distribution was examined. Among these cell lines, both DNA fragmentation (at 60 h) and G2 arrest were observed only in SW-480, analogous to the situation with VP-16 treatment (Figs. 4 and 5). A prominent increase of cells in the G2M phase of the cell cycle appeared at 48 h after irradiation in the SW-480 case.

DISCUSSION

DNA damage results in arrest of cells in the G1 and G2 phases of the cell cycle through mechanisms involving the G1 and G2 checkpoints, respectively.[5] It has been considered that these checkpoints play a protective role against DNA damage by allowing more time for DNA repair,[15,16] and the G1 checkpoint is also involved in induction of apoptosis through the p53 function in the case of failure to repair DNA damage, especially in mammalian cells.[17,18] In other studies, established cell lines known to lack p53 expression, such as human leukemia cell line HL-60,[3,4] were found to undergo apoptosis upon treatment with chemotherapeutic agents at high concentrations.[9,17] The underlying mechanism of the p53-independent apoptosis is still unclear.

In the present study, the p53 status of five human colon cancer cell lines was clarified through a yeast functional assay, and correlated with DNA damage-induced apoptosis. Two cell lines, SW-480 and COLO320, expressing no functional p53 and arrested in the G2M phase rather than the G1 phase, underwent apoptosis after both γ-irradiation and VP-16 treatment, whereas SW-620 and DID-1 cells, which express no functional p53 and showed no remarkable change in their cell cycle distributions, did not. Surprisingly, LS174T cells failed to undergo apoptosis after VP-16 treatment, even though they express wild-type p53 (Table I). These results indicate that there is a hitherto unknown apoptotic pathway in DNA-damaged colon cancer cells that is independent of p53 and may be associated with the G2 checkpoint machinery. The findings are in line with the demonstration by Strasser *et al.* that T lymphoma cells from *p53* knockout mice undergo apoptosis and arrest in the G2M phase of the cell cycle following DNA damage.[10] They speculated that a mammalian homolog of the yeast rad-9 gene may regulate induction of apoptosis via the G2 checkpoint as does the *p53* gene at the G1 checkpoint.[15,19]

It has been reported that p53-regulated genes, such as *GADD45* and *BAX*, are rapidly induced in correlation with the occurrence of apoptosis following γ-irradiation in human cell lines with a normal p53 status.[20] However, we observed no induction of apoptosis after VP-16 treatment in LS174T cells which express wild-type p53. Since several human cell lines which undergo little apoptosis after γ-irradiation despite a normal p53 status were found to show no increase in *GADD45* and *BAX* mRNA expression,[20] it is possible that the lack of apoptosis in LS174T cells brings about lack of expression of these p53 associated genes, a possibility which requires examination in future studies.

Judging from the results of cell cycle analysis following DNA damage in the present case, the reason why SW-620 and DLD-1 cells did not undergo apoptosis could be linked with a failure of the G2 checkpoint system. Previous studies have supported the concept that an accumulating loss of tumor suppressor genes on different chromosomes correlates with progression in colorectal tumorigenesis.[21,22] Although long-term passage might have caused genotypic variation in SW-620 and DLD-1 cells, resulting in lack of apoptosis induction, there is also the possibility that an important gene involved in induction of apoptosis via the G2 checkpoint following DNA damage was already mutated or deleted before establishment of these cells and that, conversely, the genes essential for p53-independent apoptosis are intact in SW-480 and COLO320 cells. We can speculate, for example, that these genes may be mammalian homologues of G2 checkpoint genes isolated in yeast, such as RAD9, RAD17, RAD24, and MEC3, although at the present the mechanisms of G2 checkpoint control in mammalian cells are still unknown.[23]

Further studies are thus needed to clarify in detail the mechanisms responsible for induction of apoptosis via G2 checkpoint control. Identification of the molecular mediators may contribute to improved effectiveness of both cancer chemotherapy and radiation therapy in the future.

(Received September 2, 1996/Accepted October 31, 1996)

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