Jpn. J. Cancer Res. **89**, 862–869, August 1998

The TdT-mediated dUTP Nick End Labeling Assay Precisely Assesses the DNA Damage in Human Tumor Xenografts

Hiroaki Kikuchi,[1, 3] Shigeki Ujiie[1] and Ryunosuke Kanamaru[2]

[1]Division of Pharmacotherapy, The Research Institute, Miyagi Cancer Center, 47-1 Nodayama, Medeshima-Shiode, Natori 981-1293 and [2]Department of Clinical Oncology, The Research Institute for Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575

Cultured HL-60, HeLa S3 and WiDr cells grown in male BALB/c *nu*/*nu* mice were studied by conventional and field-inversion DNA gel electrophoresis (FIGE), as well as by means of cytomorphological approaches, including TdT-mediated dUTP nick end labeling (TUNEL) assay. Chemosensitivity tests revealed HL-60 to be sensitive to vindesine (VDS), and HeLa S3 and WiDr to mitomycin C (MMC). Although VDS-treated HL-60 exhibited condensation of chromatin and a DNA ladder, MMC-exposed HL-60 cells showed apoptotic figures without typical DNA ladders. With MMC-treated WiDr cells, neither DNA ladders nor apoptotic figures were observed. Cells characterized by chromatin condensation were TUNEL-positive in both treated and untreated cases with the exception of the MMC-treated WiDr case, in which many TUNEL-positive cells were observed without cytomorphological changes. On FIGE, DNA fragments of approximately 50, 300 and 400 kbp were detected in groups treated with both effective and ineffective drugs, as well as in untreated controls. Furthermore, change of the time parameters in FIGE resulted in different sizes (550 and 850 kbp) of DNA fragments. These findings indicate that i) cell death is not always detectable in terms of apoptotic figures or DNA oligonucleosomal fragmentation, ii) only the TUNEL assay is a reliable tool to detect DNA damage and, iii) FIGE does not provide accurate size profiles of macromolecular DNA fragments.

Key words: DNA damage — Cell death — DNA gel electrophoresis — Human tumor xenograft — Nude mouse

A variety of approaches have been utilized to investigate the modes of DNA damage leading to cell death. In the past, apoptosis has been characterized biochemically by the production of DNA oligonucleosomal fragments. However, this now appears to be an oversimplification.[1)] Investigators[2–7)] using pulsed-field gel electrophoresis have observed DNA fragments of 50 and/or 300 kbp both with and without oligonucleosomal fragments in cells showing morphological features of apoptosis. Macromolecular DNA breaks are considered to be generated by activation of topoisomerase II,[2)] whereas endonucleases are responsible for production of oligonucleosomal fragments. Changes in the integrity of DNA can be evaluated by using pulsed-field gel electrophoresis, which allows analysis of DNA with lengths up to 10 Mbp.[8)] The equipment for this purpose is very expensive, however, whereas field-inversion gel electrophoresis (FIGE) requires only a pulse controller in addition to a power supply and gel chamber, which are used for conventional DNA gel electrophoresis, and can distinguish DNA fragments up to 1 Mbp.[9)]

Up to the present, investigators[2–9)] have mainly applied pulsed-field gel electrophoresis for detecting DNA damage *in vitro*. Although *in vitro* systems for studying cancer are less time-consuming and expensive than those *in vivo*, there is difficulty in deciding drug concentrations for testing chemosensitivity.[10–13)] In contrast, human tumor xenograft systems do not permit overdosage due to the existence of maximal tolerable doses,[14, 15)] and hence are suitable for studies of cancer chemotherapy.

As an alternative assay method for apoptosis or programmed cell death, the TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay has been introduced.[16)] The DNA fragmentation end-point is also typical of necrotic cells,[7, 17)] so that care must be taken with interpretation, but this assay has the marked advantage of providing *in situ* information. In the present study, representative human tumor cell lines were transplanted into nude mice and chemosensitivity tests were carried out to select effective agents. In order to compare the modes of DNA damage among cell lines and anti-cancer agents, samples of the tumors were taken for conventional and field-inversion DNA gel electrophoresis as well as cytomorphological examination, including *in situ* TUNEL assays. The modes of DNA alteration of tumor cells in the region of "central necrosis" and normal cells of untreated mice were also investigated.

[3] To whom correspondence and requests for reprints should be addressed.

MATERIALS AND METHODS

Cell culture HeLa S3, WiDr and HL-60 cells were purchased from Dai-Nippon Seiyaku Co., Ltd. (Tokyo) and maintained in Dulbecco's modified Eagle's medium (Nissui, Tokyo) or RPMI1640 medium (Nissui) in the HL-60 case, both containing penicillin G (5×10^3 U/ml, Gibco BRL, Gaithersberg, MD), streptomycin (5 mg/ml, Gibco BRL) and 10% fetal bovine serum (Bioserum, Victoria, Australia). Cells were cultured at 37°C under 5% CO_2 in a fully humidified atmosphere.

Animals, tumor implantation and chemosensitivity test Male BALB/c *nu*/*nu* mice, five weeks old, were purchased from Charles River Japan Inc. (Yokohama) and maintained under specific pathogen-free conditions in a laminar air flow system at a constant temperature (25±1°C) and humidity (55±5%). They received food and water *ad libitum*. Tumor cells (1×10^7) were injected into subcutaneous tissue of the mice and after about 30 days, tumors which had grown to approximately 1 cm in diameter were removed. After trimming, the tumors were minced into fragments of 2-mm diameter and transplanted into subcutaneous tissue of 30–40 mice using a trocar. When the estimated tumor weight ($L\times W^2\times0.5$) reached 100–150 mg, mice were divided at random into groups of five animals and the tumor sizes and the body weights were noted.

Anti-cancer drugs were administered at the MTD level for male BALB/c *nu*/*nu* mice.[15] They were adriamycin (Kyowa Hakko Co., Tokyo), 8 mg/kg; mitomycin C (MMC; Kyowa Hakko Co.), 6 mg/kg; cisplatinum (Bristol-Myers Squibb Co., Tokyo), 8 mg/kg; cyclophosphamide (Shionogi Co., Osaka), 120 mg/kg; nimustine (Sankyo Co., Tokyo), 30 mg/kg; etoposide (Bristol-Myers Squibb Co.), 50 mg/kg; vindesine (Shionogi Co.), 3 mg/kg; vincristine (Shionogi Co.), 1 mg/kg and 5-fluorouracil, 50 mg/kg (q4d×3, Kyowa Hakko Co.). All agents were given intraperitoneally except for adriamycin, administered intravenously. Tumor diameters and body weights were monitored for 21 days after initial treatment and then inhibition rates (IR) were calculated by using the formula; $IR=(1-T/C)\times100$ (%), where T and C are the mean tumor weights of the treated and untreated groups. The response to anti-cancer agents was considered to be good when the IR was more than 58%. At the termination, mice were killed under diethyl ether anesthesia and tumors were removed. Tissue was processed for morphological examination, including *in situ* TUNEL assays and DNA analysis by gel electrophoresis.

***In situ* TUNEL assay** In the present study, "ApopTag Direct," an apoptosis detection kit (Oncor, Gaithersburg, MD) was used to perform TUNEL assays following the recommended protocol. Briefly, histological sections were deparaffinized and protein was digested with "Protein Digesting Enzyme" (Oncor, 20 μg/ml) for 15 min at room temperature followed by staining steps. Positive control slides were prepared by nicking DNA with DNase I (Sigma Chemical Co., St. Louis, MO). For observation, a fluorescence microscope with a 515–565 nm (green) and a 575–640 nm (red) band-pass filter was employed.

Conventional electrophoresis of cellular DNA Samples were minced in ice-cold Tris-buffered saline and homogenized with a laboratory homogenizer (Kinematica, Littau, Switzerland) at 5,000 rpm for 30 s. They were filtered and washed twice with ice-cold Tris-buffered saline followed by resuspension in 500 μl of lysis buffer containing 500 m*M* Tris-HCl (pH 9.0), 2 m*M* EDTA, 10 m*M* NaCl, 1% sodium dodecyl sulfate (SDS) and 1 mg/ml proteinase K (Wako Chemical, Osaka). After incubation at 50°C for

Table I. Inhibition Rates (IR) for Human Tumor Xenografts and Maximal Weight Loss in BALB/c *nu*/*nu* Mice

Drug	HL-60 (%)	HeLa S3 (%)	WiDr (%)
Adriamycin	21.7±9.4[a] (3.8[b])	34.9±3.7 (9.1)	12.3±7.1 (5.4)
Cisplatin	39.7±11.5 (4.4)	41.9±17.2 (7.0)	38.5±9.4 (6.8)
Mitomycin C	30.8±5.7 (17.5)	83.0[c]±9.4 (16.2)	87.7[c]±10.1 (18.7)
Cyclophosphamide	65.8[c]±4.9 (2.3)	52.7±11.4 (3.7)	23.1±10.5 (3.0)
Nimustine	2.03±1.2 (4.2)	24.5±6.7 (0.8)	33.8±8.9 (1.5)
5-Fluorouracil	47.1±14.3 (1.7)	50.6±10.6 (1.7)	38.5±12.4 (2.5)
Etoposide	38.6±4.0 (7.6)	n.t.	n.t.
Vincristine	94.8[c]±1.9 (10.2)	n.t.	n.t.
Vindesine	99.7[c]±0.1 (18.5)	n.t.	n.t.

a) IR (for details, see "Materials and Methods") are represented as mean±SE (n=5).
b) Maximal weight loss is the difference of the lowest mouse body weight during the assay period (21 days) from the initial weight.
c) IR over 58% (defined as effective for the study).
n.t.: not tested.

20 h, samples were extracted twice with phenol, once with phenol-chloroform and finally with chloroform, and aliquots (1 μg) were loaded on 1.5% agarose (Nippon Gene, Tokyo) gels. Electrophoresis was performed for 3 h at 6 V/cm in 0.5× TBE buffer. Gels were stained with 0.5 μg/ml ethidium bromide (30 min) and photographed under UV light (312 nm).

FIGE FIGE was carried out using a programmable power inverter, PPI-200 (MJ Research, Watertown, MA). Agarose plugs containing 5×10^5 cells were prepared and digested in buffer containing 0.5 *M* EDTA (pH 9.0), 1% sodium *N*-lauroyl sarcosinate (Wako Chemical) and 1 mg/ml proteinase K (Wako Chemical) at 50°C for 48 h. Runs were performed in 1% pulsed-field grade agarose (Nippon Gene) at 5 V/cm in 0.5× TBE buffer (10°C) using a horizontal gel chamber GNA-200 (Pharmacia Biotech, Uppsala, Sweden) with a 15 min continuous forward pulse followed by 0.3 s forward to 0.1 s reverse (3:1 ratio) with a ramp factor of 0.1 s applied, increasing the forward pulse time up to 30 s and reverse phase to 10 s. Under these conditions, DNA fragments ranging in size from 50 to 400 kbp could be resolved within 48 h. DNA fragments ranging from 50 to 850 kbp were separated with a 75 s maximal forward pulse time and reverse phase of 25 s.

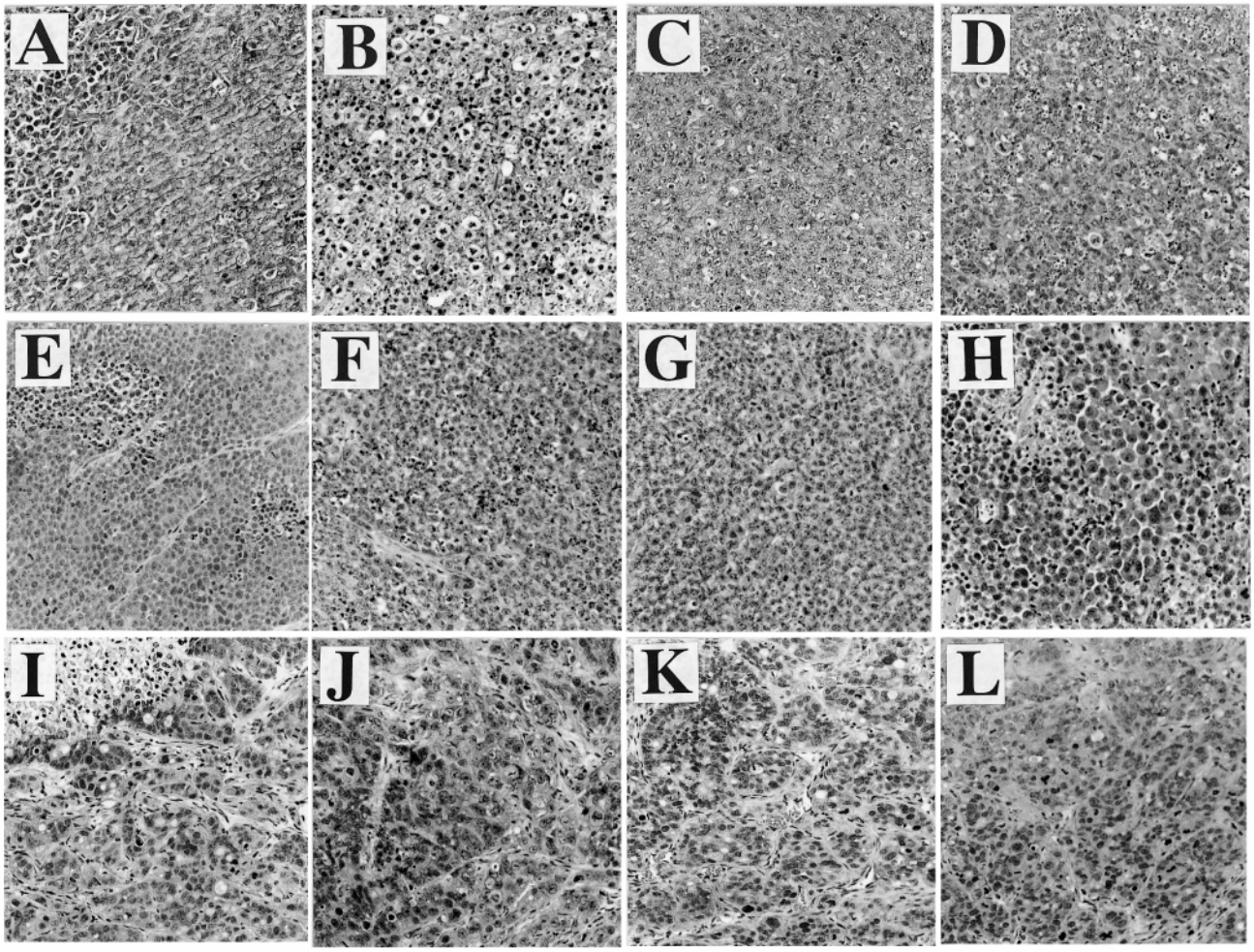

Fig. 1. Light microscopic appearance of HL-60, HeLa S3 and WiDr cells as human tumor xenografts in nude mice treated with anticancer agents (for details, see "Materials and Methods," H-E, ×100). In all the untreated controls, cells with shrinkage of cytoplasm, chromatin condensation and nuclear fragmentation are seen in a mass in the areas of "central necrosis," as well as scattered in the intact tumor tissue. A–D, HL-60 cells (A, control; B, VDS at 48 h; C, ADM at 48 h; D, MMC at 48 h). Apoptotic figures are marked with VDS, but less marked with MMC and lacking in the ADM-treated specimen. E–H, HeLa S3 cells (E, control; F, CDDP at 48 h; G, ADM at 48 h; H, MMC at 48 h). Apoptotic figures are apparent after MMC and CDDP-treatment, but not in the ADM-treated specimen. I–L, WiDr cells (I, control; J, CDDP at 48 h; K, ADM at 48 h; L, MMC at 48 h). Morphological changes are minimal in all cases. Apoptotic figures are seen only in the area of "central necrosis."

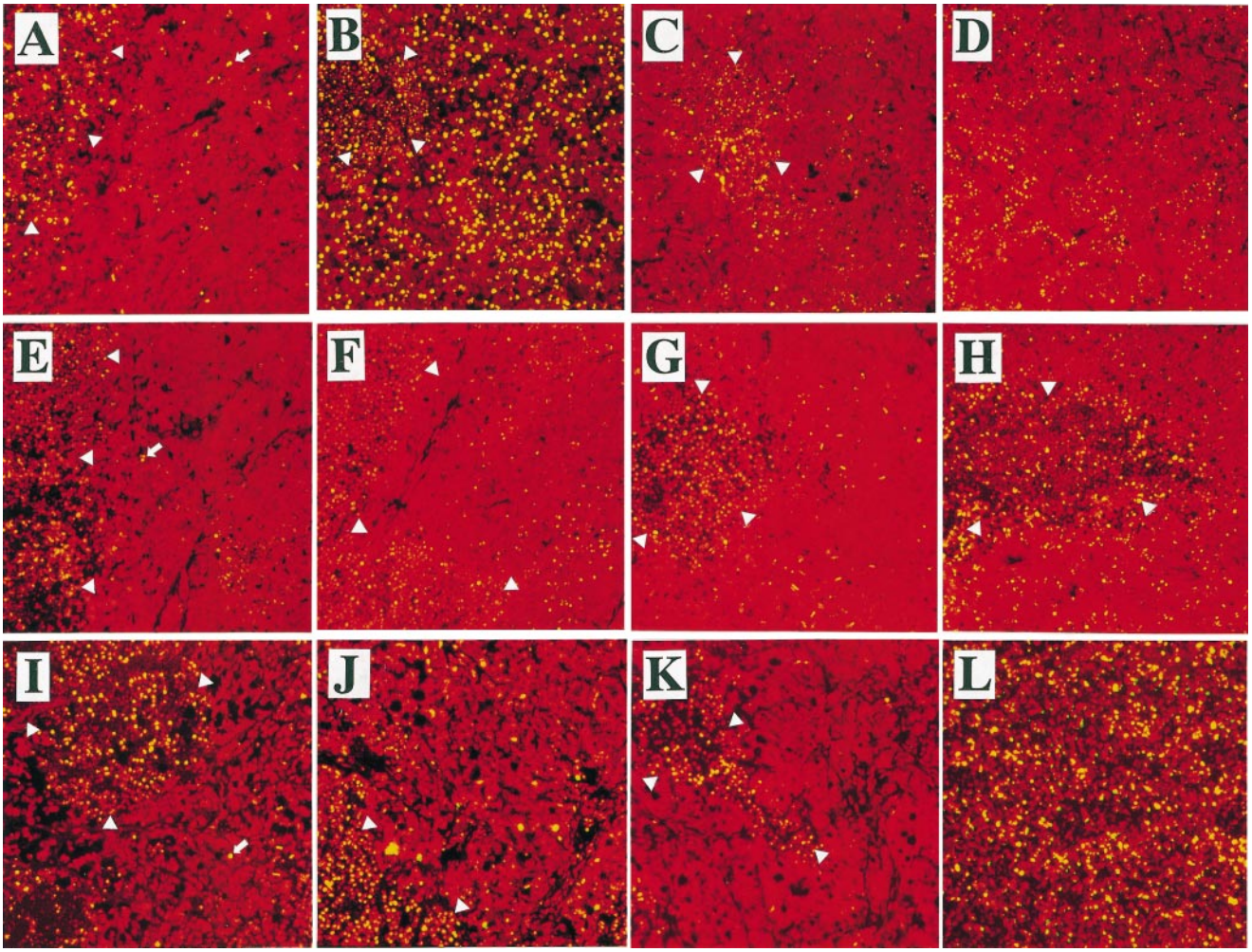

Fig. 2. *In situ* TUNEL assay results for HL-60, HeLa S3 and WiDr cells (for details, see "Materials and Methods," ×100). In all the control cases, cells in the area of "central necrosis" are markedly positive in a mass (△). Some positive cells are seen scattered in the intact tumor tissue (↟). Positive cells in the "central necrosis" of the treated cases are also indicated by arrows (△). A–D, HL-60 cells (A, control; B, VDS at 48 h; C, ADM at 48 h; D, MMC at 48 h). VDS-treated samples show diffuse positive cells, although they are fewer in the MMC or ADM-treatment case. E–H, HeLa S3 cells (E, control; F, CDDP at 48 h; G, ADM at 48 h; H, MMC at 48 h). MMC and CDDP-treated samples show more positive cells than in the ADM-treatment case, but fewer than in the HL-60 case in general. I–L, WiDr cells (I, control; J, CDDP at 48 h; K, ADM at 48 h; L, MMC at 48 h). Diffuse positive cells are evident only in the MMC-treated sample except in the area of "central necrosis."

Molecular weight markers ("PULSE MARKER," 50–1,000 kb) were purchased from Sigma Chemical Co. Gels were stained with 0.5 μg/ml ethidium bromide (30 min) and photographed under UV light (312 nm).

Alkaline gel electrophoresis Gels containing 1.5% agarose, 50 m*M* NaCl and 1 m*M* EDTA were prepared and soaked in alkaline buffer (20 m*M* NaOH/4 m*M* EDTA) over 30 min before runs. DNA samples (1 μg) were applied immediately after being mixed with loading buffer (25% glycerol/0.05% bromocresol green/50 m*M* NaOH/1 m*M* EDTA) and run at 5 V/cm for 15 min followed by 2 V/cm for 8 h. Gels were stained with 0.5 μg/ml ethidium bromide (30 min) and photographed under UV light (312 nm).

RESULTS

Chemosensitivity testing Data for anti-tumor activities of the different drugs against HL-60, HeLa S3 and WiDr cells in nude mice are summarized in Table I. Among the

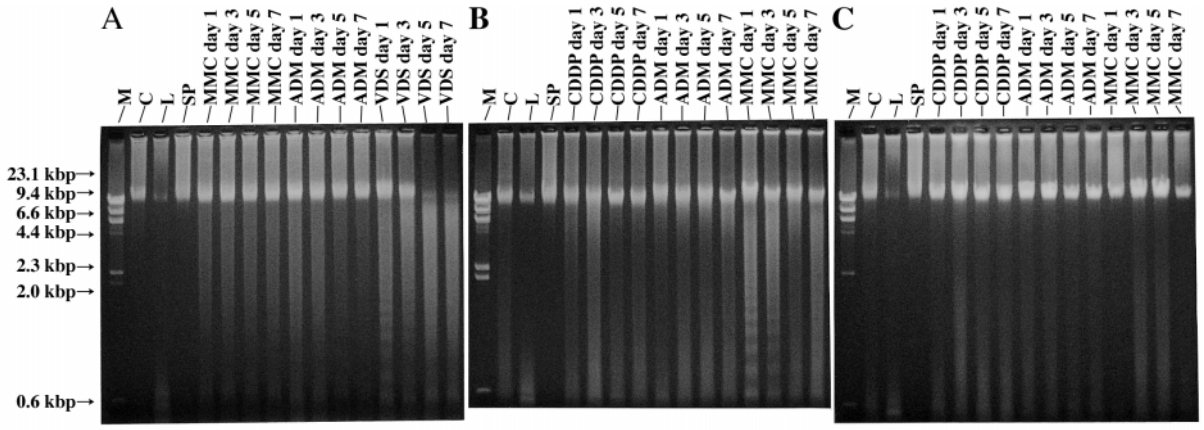

Fig. 3. Conventional agarose gel electrophoresis of the DNA from HL-60, HeLa S3 and WiDr cells in nude mice (for details, see "Materials and Methods"). The marker lane (M) shows DNAs of 23,130, 9,416, 6,557, 4,361, 2,322, 2,027 and 564 bp. C, control untreated cells; L, liver cells of an untreated mouse; SP, spleen cells of an untreated mouse. A: HL-60 cells, 1 to 3 days after treatment with VDS, typical ladder patterns are evident. Note the lack of a DNA ladder in ADM and MMC-treated samples. B: HeLa S3 cells, a ladder pattern is present after MMC-treatment and only smears are apparent in ADM and CDDP-treated samples. C: WiDr cells, only slight smears are apparent in all groups.

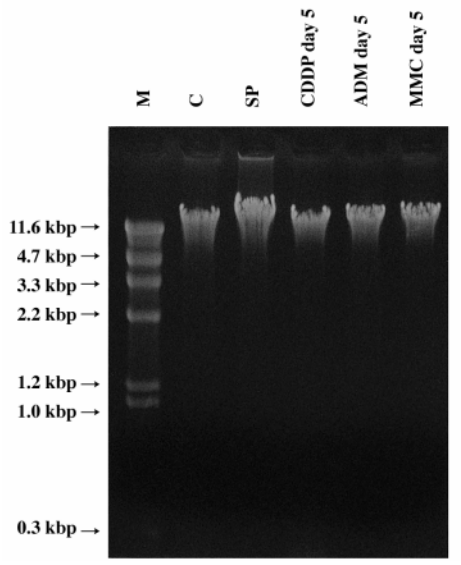

Fig. 4. Alkali gel electrophoresis of WiDr cells in nude mice. The marker lane (M) shows DNAs of 11,565, 4,708, 3,279, 2,181, 1,161, 1,014 and 282 bp. C, control untreated cells; SP, spleen cells of an untreated mouse. Note the lack of DNA ladders.

agents tested in the present study, cyclophosphamide, vincristine and vindesine (VDS) were effective for HL-60, and only MMC for HeLa S3 and WiDr. Adriamycin (ADM) and cisplatinum (CDDP) were ineffective. Accordingly, HL-60 was treated with VDS, ADM and MMC and HeLa S3 and WiDr were treated with MMC, ADM and CDDP for the following experiments. Body weight loss was less than 20% in the assay period (21 days).

Morphology and *in situ* TUNEL assay results Morphological findings for HL-60, HeLa S3 and WiDr cells are represented in Fig. 1. In all cell lines, condensation of chromatin and nuclear fragmentation were observed in areas of so-called "central necrosis." In HL-60, chromatin condensation and nuclear fragmentation were prominent in the VDS-treated group, but were less so with MMC-treatment and were rare or lacking with ADM. In HeLa S3, these features were similarly noted for MMC and CDDP-treatments, but were rare or lacking with ADM. In WiDr, on the other hand, minimal morphological changes were observed in both treated and non-treated groups except for areas of "central necrosis," which showed condensation of chromatin. Comparison with the results of the TUNEL assays (Fig. 2) showed apoptotic figures to contain a multitude of 3′-OH DNA ends. However, in the MMC-treated WiDr case, diffuse TUNEL-positive cells were also observed without apoptotic figures on light microscopy. TUNEL-positive cells were observed in the treated groups as well as in the areas of the "central necrosis" in untreated controls.

DNA fragments in conventional, field-inversion and alkali gel electrophoresis With conventional DNA gel electrophoresis (Fig. 3), oligonucleosomal fragments were apparent in samples of VDS-treated, but not in ADM- or MMC-treated HL-60 cells. Although the control specimens showed only a slight degree of smearing, this was not attributable to a technical error, because mouse spleen DNA showed no smear pattern. Hence the DNA smears

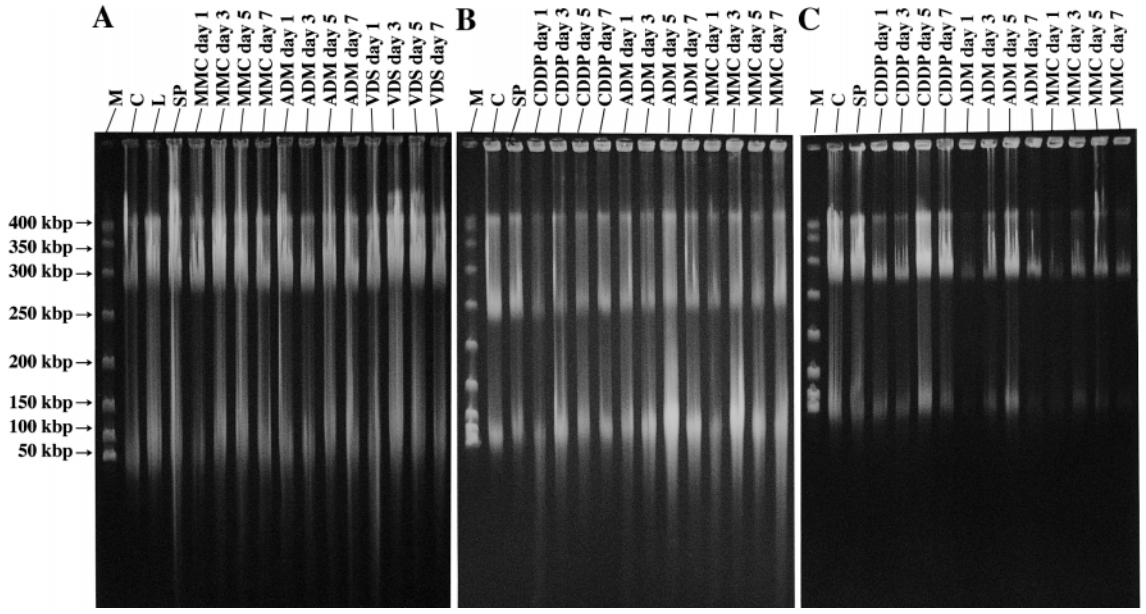

Fig. 5. FIGE of DNA from HL-60, HeLa S3 and WiDr cells grown in nude mice (for details, see "Materials and Methods"). The marker lane (M) shows DNAs of 50, 100, 150, 200, 250, 300, 350 and 400 kbp ("PULSE MARKER," 50–1,000 kb from Sigma Chemical Co.). C, control untreated cells; L, liver cells of an untreated mouse; SP, spleen cells of an untreated mouse. A: HL-60 cells. B: HeLa S3 cells. C: WiDr cells. Untreated tumor cells of all lines show DNA fragments of approximately 50, 300 and 400 kbp, without any influence of effective drugs (i.e. VDS for HL-60 and MMC for HeLa S3 and WiDr). Mouse spleen and liver cells show DNA fragments of the same sizes.

in the non-treated control were considered to be derived from cells in the area of the "central necrosis." Since WiDr cells did not show DNA ladders with any drug, alkali gel electrophoresis was performed to detect single-strand DNA breaks. However, in this assay too, no DNA ladders were detected (Fig. 4).

Portions of the same samples were subjected to FIGE (Fig. 5). Similar sizes of DNA fragments with molecular weights of approximately 50, 300 and 400 kbp were observed for all three cell lines. There was no influence of anti-cancer agents, and furthermore, untreated controls showed DNA fragments of the same sizes. Similar results were obtained for spleen and liver cells of untreated mice.

Mouse liver and spleen cells, and cultured HL-60, HeLa S3 and WiDr cells were then prepared for agarose plugs and digested at 50°C for 48 h in buffer containing 0.5 *M* EDTA (pH 9.0), 1 or 10 mg/ml proteinase K (Wako Chemical) and 1 or 10% sodium *N*-lauroyl sarcosinate (Wako Chemical). FIGE was then performed at a 3:1 ratio with 30 or 75 s maximal forward pulse time (Fig. 6). DNA fragments of approximately 300 and 400 kbp were recognized in all samples, but especially in spleen cells with a 30 s maximal forward pulse time. In contrast, with a 75 s maximal forward pulse time, DNA fragments of approximately 550 and 850 kbp were seen.

DISCUSSION

A number of investigators[2–7] have noted that cells showing apoptotic figures contained DNA fragments of 50–300 kbp with or without oligonucleosomal fragments. Most of these studies used *in vitro* systems, in which all cells are considered to grow exponentially and to be immortal in the absence of cytotoxicity. There are difficulties in deciding appropriate drug concentrations when chemosensitivity is tested *in vitro*,[10–13] because high concentrations could modify the mode of DNA damage. For instance, high doses of ethyl alcohol resulted in DNA smears in HL-60 cells, instead of the DNA ladders observed at low doses *in vitro*.[18, 19]

Human tumor xenograft systems for studies of cancer,[14, 15] in contrast, do not permit overdosage due to the limit of the maximal tolerable dose and thus provide good models for chemotherapy. In solid tumors, spontaneous cell death, which is generally called "central necrosis," is attributed mainly to an insufficiency of neovascularization. Recent studies have revealed that the cell death is of apoptotic type.[20] This can only be investigated *in vivo*. In the present study, tumors taken from mice were carefully trimmed to eliminate areas of "central necrosis." However, cells showing apoptotic figures and

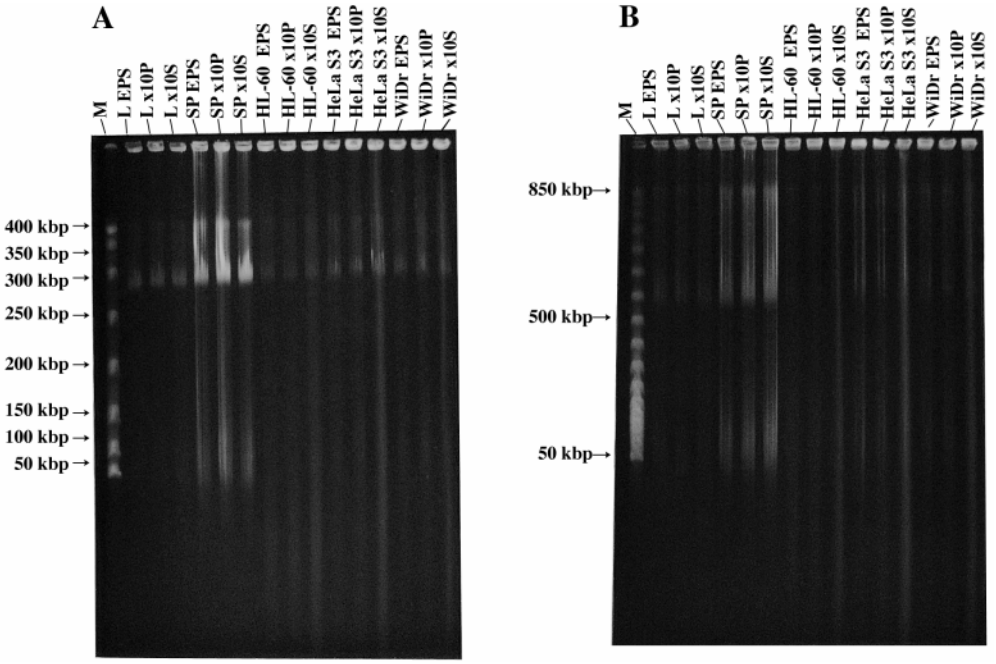

Fig. 6. FIGE of DNA from mouse liver and spleen cells and cultured HL-60, HeLa S3 and WiDr cells (for details, see "Materials and Methods"). The marker lane (M) shows DNAs of 50, 100, 150, 200...kbp ("PULSE MARKER," 50–1,000 kb from Sigma Chemical Co.). L, liver cells of an untreated mouse; SP, spleen cells of an untreated mouse; EPS, samples digested in buffer containing 0.5 *M* EDTA (pH 9.0), 1 mg/ml proteinase K and 1% sodium *N*-lauroyl sarcosinate; ×10P, containing 10 mg/ml proteinase K in digestion buffer, otherwise the same; ×10S, containing 10% sodium *N*-lauroyl sarcosinate in digesting buffer, otherwise the same. A: 30 s maximal forward pulse time. DNA fragments of approximately 300 and 400 kbp are apparent, especially in the spleen cell case. Augmentation of proteinase K or sodium *N*-lauroyl sarcosinate was without effect. B: 75 s maximal forward pulse time. DNA fragments of approximately 550 and 850 kbp are recognizable in every sample. Augmentation of proteinase K or sodium *N*-lauroyl sarcosinate was also without effect.

TUNEL positivity were seen in all treated samples of tumor tissue, as well as in untreated controls. The latter showed slight DNA smears in conventional gel electrophoresis and DNA fragments of molecular weights of 50, 300 and 400 kbp in FIGE, presumably because not all spontaneously dead cells could be removed by the trimming procedure.

Drugs which act as inhibitors of topoisomerase II have been reported to produce a cleavable complex which splits DNA at 50–300 kbp.[2, 21] In the present study, untreated xenograft tumors contained DNA fragments with molecular weights of approximately 50, 300 and 400 kbp on FIGE, probably due to inactivation of the topoisomerase II in spontaneously dead cells. Compared with ineffective drugs or untreated controls, however, effective drugs did not change the size of the macromolecular DNA fragments, with or without oligonucleosomal fragmentation.

Furthermore, spleen or liver cells of the untreated mice showed no DNA damage on conventional gel electrophoresis, but DNA fragments of approximately 300 and 400 kbp were seen on FIGE. There were no TUNEL-positive cells in the spleen or liver of untreated mice (data not shown). Augmentation of proteinase K or sodium *N*-lauroyl sarcosinate to the digesting buffer did not change the fragment sizes. Change of the maximal forward pulse time on FIGE gave different ranges of resolution of the DNA fragments. When the marker DNA fragments of 50–400 kbp were resolved (30 s maximal forward pulse time), approximately 50, 300 and 400 kbp fragments were separated from samples. In contrast, 75 s maximal forward pulse time resolved the marker DNA fragments of 50–850 kbp, and fragments of approximately 550 and 850 kbp were detected in the same samples. Since the change in timing parameters of the pulse controller gave different outcomes, the estimation of macromolecular sizes of DNA fragments detected on FIGE can not be concluded to be always reliable, so that this approach is not informative. Some other method for pulsed-field gel electrophoresis, e.g. contour-clamped homogenous electric field gel electrophoresis,[22] might solve this problem. In any case, the macromolecular DNA sizes assessed with FIGE were not reliable in this study.

While MMC was an effective agent against WiDr cells in the study, neither DNA ladders, massive smears nor apoptotic figures were observed. In the TUNEL assay, however, many positive cells were apparent 48 h after MMC-treatment. These findings suggest that WiDr cells killed by MMC contained only high-molecular-weight DNA fragments and did not show typical apoptotic figures. If the MMC-treated DNA of WiDr cells were broken into low-molecular-weight fragments, apoptotic figures would be seen, as in cells in the areas of "central necrosis." Nevertheless, this type of DNA damage was associated with apparent tumor reduction in nude mice. Neither the light microscopic findings nor the results of DNA gel electrophoresis could predict the cytotoxicity of MMC against WiDr cells. At present, we therefore consider that the TUNEL assay may be the most accurate tool for detecting DNA damage and chemosensitivity, although it is difficult to carry out a quantitative analysis in an *in situ* study.

(Received April 21, 1998/Revised June 3, 1998/Accepted June 10, 1998)

REFERENCES

1) Collins, R. J., Harmon, B. V., Gobe, G. C. and Kerr, J. F. Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int. J. Rad. Biol.*, **61**, 451–453 (1992).
2) Walker, P. R., Smith, C., Youdale, T., Leblanc, J., Whitfield, J. F. and Sikorska, M. Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res.*, **51**, 1078–1085 (1991).
3) Walker, P. R., Kokileva, L., LeBlanc, J. and Sikorska, M. Detection of the initial stages of DNA fragmentation in apoptosis. *Biotechniques*, **15**, 1032–1040 (1993).
4) Brown, D. G., Sun, X. M. and Cohen, G. M. Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. *J. Biol. Chem.*, **268**, 3037–3039 (1993).
5) Oberhammar, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R. and Sikorska M. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.*, **12**, 3679–3684 (1993).
6) Lagarkova, M. A., Iarovaia, O. V. and Razin, S. V. Large-scale fragmentation of mammalian DNA in the course of apoptosis proceeds via excision of chromosomal DNA loops and their oligomers. *J. Biol. Chem.*, **270**, 20239–20241 (1995).
7) Takei, T., Araki, A., Watanabe, H., Ichinose, A. and Sendo, F. Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis. *J. Leukoc. Biol.*, **59**, 229–240 (1996).
8) Schwartz, D. C. and Cantor, C. R. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*, **37**, 67–75 (1984).
9) Carle, G. F., Frank, M. and Olson, M. V. Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science*, **232**, 65–68 (1986).
10) Smit, E. F., de Vries, E. G., Timmer-Bosscha, H., de Leij, L. F., Oosterhuis, J. W., Scheper, R. J., Weening, J. J., Postmus, P. E. and Mulder, N. H. *In vitro* response of human small-cell lung cancer cell lines to chemotherapeutic drugs; no correlation with clinical data. *Int. J. Cancer*, **51**, 72–77 (1992).
11) Bellamy, W. T. Prediction of response to drug therapy of cancer. A review of *in vitro* assays. *Drugs*, **44**, 690–708 (1992).
12) Durand, R. E. The influence of microenvironmental factors during cancer therapy. *In Vivo*, **8**, 691–702 (1994).
13) Kondo, T. Problems in chemosensitivity testing. *Jpn. J. Cancer Chemother.*, **22**, 592–598 (1995) (in Japanese).
14) Nishiyama, M., Saeki, S., Aogi, K., Hirabayashi, N. and Toge, T. Relevance of DT-diaphorase activity to mitomycin C (MMC) efficacy on human cancer cells: differences in *in vitro* and *in vivo* systems. *Int. J. Cancer*, **53**, 1013–1016 (1993).
15) Kondo, T., Imaizumi, M., Taguchi, T., Abe, O., Hattori, T., Wakui, A. and Kitano, M. A model for the sensitivity determination of anticancer agents against human cancer using nude mice. *Jpn. J. Cancer Chemother.*, **14**, 680–686 (1987) (in Japanese).
16) Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.*, **119**, 493–501 (1992).
17) Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H. P., Toyka, K. V. and Lassmann, H. Differentiation between cellular apoptosis and necrosis by the combined use of *in situ* tailing and nick translation techniques. *Lab. Invest.*, **71**, 219–225 (1994).
18) Lennon, S. V., Martin, S. J. and Cotter, T. G. Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Prolif.*, **24**, 203–214 (1991).
19) Kikuchi, H., Ujiie, S., Wakui, A., Yokoyama, A. and Kanamaru, R. Features of DNA oligonucleosomal fragmentation in human tumor cell lines and its detection by flow cytometry: utility and limitations. *Jpn. J. Cancer Res.*, **88**, 56–63 (1997).
20) Wu, J. Apoptosis and angiogenesis: two promising tumor markers in breast cancer. *Anticancer Res.*, **16**, 2233–2239 (1996).
21) Roy, C., Brown, E. L., Little, J. E., Valentine, B. K., Walker, P. R., Sikorska, M., Leblanc, J. and Chaly, N. The topoisomerase II inhibitor teniposide (VM-26) induces apoptosis in unstimulated mature murine lymphocytes. *Exp. Cell Res.*, **200**, 416–424 (1992).
22) Chu, G., Vollrath, D. and Davis, R. W. Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science*, **234**, 1582–1585 (1986).