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Analysis of DNA Fragmentation in Human Uterine Cervix Carcinoma HeLa S_3 Cells Treated with Duocarmycins or Other Antitumor Agents by Pulse Field Gel Electrophoresis

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Pulse field gel electrophoresis using a contour-clamped homogeneous electric field was applied for the analysis of DNA-fragmenting activity of antitumor agents towards human uterine cervix carcinoma HeLa S_3 cells. Duocarmycins (DUMs), novel antitumor antibiotics with ultrapotent cell growth-inhibitory activities, caused DNA fragmentation at 10 times their IC_{50} values at 2 h exposure. At 100 times their IC_{50} values, the size of the smallest fragments was about 245 kilobase pairs (kbp). DUMA, DUMB1 and DUMB2 exhibited similar DNA fragmentation patterns, suggesting similar action mechanisms. DNA fragmentation was also detected in cells treated with radical producers, intercalators and topoisomerase inhibitors. Two bands of about 1800 and 1500 kbp were commonly detected in the cells treated with DUMs and these agents. In addition, fragments of about 900 kbp were detected in the cells treated with a topoisomerase inhibitor, 4′-(9-acridinylamino)methanesulfon-*m*-anisidine, and fragments in the broad size range between 700 and 245 kbp in the cells treated with radical producers, bleomycin and neocarzinostatin. DUMs showed a characteristic DNA fragmentation pattern, since both types of fragments induced by the topoisomerase inhibitor and the radical producers were simultaneously detected, suggesting a novel mode of interaction with DNA. DNA-crosslinking agents and mitotic inhibitors did not induce DNA fragmentation under these conditions. The pulse field gel electrophoresis is potentially useful for characterizing DNA-cleaving activity of various antitumor agents at the cellular level.

Key words: Duocarmycin — HeLa S_3 cell — DNA fragmentation — Pulse field gel electrophoresis

The primary action site of many antitumor agents is DNA, and the mechanisms involved have been investigated by means of various methods. Among them, the alkaline elution method and the alkaline sucrose density gradient method have many advantages for detecting DNA strand breakage at the cellular level.[1-6] Both single and double strand breaks of DNA are detectable by these two methods, by adjusting the pH of the lysis buffer to alkaline and neutral, respectively. However, these methods need considerable technical skill, and it is difficult to handle many DNA samples. Recently the method of agarose gel electrophoresis of DNA has been greatly improved by applying CHEF[2] apparatus.[7] This method enables the analysis of DNA fragments much larger than 50 kbp, and is applicable to the analysis of DNA extracted from cells treated with antitumor agents.[8,9] With this CHEF method, DNA is usually analyzed under neutral, but not alkaline, conditions, so the DNA fragments formed by double strand breaks are supposed to be detectable. DUMs are novel antitumor antibiotics with strong antitumor activity against various tumor cells *in vitro* and *in vivo*.[10] Among them, DUMA, DUMB1 and DUMB2 exhibited very potent cell growth-inhibitory activity, although their structures are different (Fig. 1). We previously reported that DUMB1 induced DNA fragmentation in human uterine cervix carcinoma HeLa S_3 cells using CHEF apparatus.[10]

In this report, we examined DNA fragmentation patterns in DUMA-, DUMB1- and DUMB2-treated HeLa S_3 cells using CHEF apparatus, and furthermore, the DNA fragmentation patterns were compared with those caused by various other antitumor drugs.

MATERIALS AND METHODS

Chemicals Duocarmycin A (DUMA), duocarmycin B_1 (DUMB1) and duocarmycin B_2 (DUMB2) were produced and purified as reported previously.[11,12] m-AMSA was kindly provided by Dr. Tsuruo, Cancer Chemotherapy Center, Tokyo, and camptothecin by Dr. Nakano, Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Tokyo. Adriamycin, epirubicin, MMC and vinorelbine (KW-2307) were obtained from Kyowa Hakko Kogyo Co.; actinomycin D from Ban-yu Pharm. Co., Tokyo; mitoxantrone from Takeda Chem. Ind., Osaka;

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[2] Abbreviations: CHEF, contour-clamped homogeneous electric field; DUM, duocarmycin; IC_{50}, concentration required for 50% growth inhibition; kbp, kilobase pairs; m-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidine; MMC, mitomycin C; VP-16, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidine-β-D-glucopyranoside).

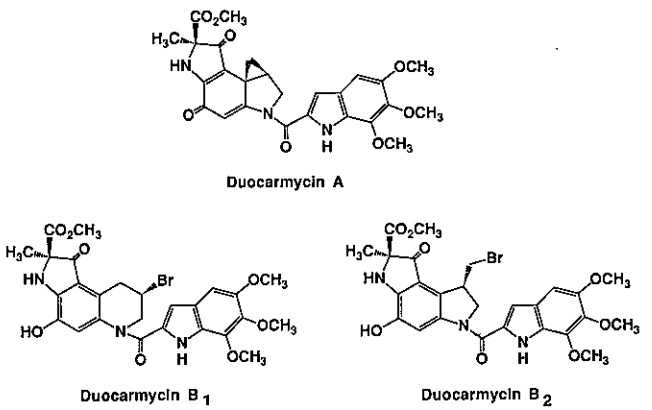

Fig. 1. Structure of DUMs.

bleomycin and VP-16 from Nippon Kayaku Co., Tokyo; neocarzinostatin from Yamanouchi Pharm. Co., Tokyo; cisplatin from Sigma Chemical Co., St. Louis, Mo.; and vincristine from Shionogi Pharm. Co., Osaka.

Cell culture Human uterine cervix carcinoma HeLa S_3 cells were cultured in monolayer in Eagle's minimal essential medium (MEM, Nissui Pharm. Co., Tokyo) supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.) at 37°C under a humidified atmosphere containing 5% CO_2 in air, and used in the exponential growth phase.

Cell growth-inhibitory activity HeLa S_3 cells (2×10^4/well) were precultured for 24 h in 24-well multidishes (Nunc, Roskilde, Denmark) containing 0.75 ml of the culture medium as described above. Then the cells were treated with a test compound for 1 h, washed, and further incubated for 71 h in the culture medium. The cell number was counted by using a Toa micro-cell counter (Toa Medical Electronics Co., Hyogo) according to the method previously reported.[13] Growth-inhibitory activity of each compound was expressed as IC_{50} (concentration required for 50% growth inhibition) value.

DNA preparation HeLa S_3 cells (9×10^6/30 ml of culture medium) precultured in a plastic flask for 24 h were treated with each compound for 2 h. The culture supernatant was discarded, and the monolayer cells were washed with Dulbecco's phosphate-buffered saline (Ca^{2+}-, Mg^{2+}-free, pH 7.2), treated with 0.02% (w/v) disodium EDTA, and centrifuged. The cell pellet was suspended in 50 m*M* disodium EDTA, mixed with an equal volume of 1.0% (w/v) low-temperature-melting agarose (Japan Bio Rad Co., Tokyo), poured into a sample-plug caster (Japan Bio Rad Co.), and left at 4°C for 60 min to solidify. The cells in the agarose block were treated with lysis buffer (pH 8.0, 10 m*M* Tris-base, 500 m*M* disodium EDTA, 1% (w/v) Sarkosyl and 1 mg/ml proteinase K (Boehringer Mannheim Yamanouchi Co., Tokyo)) at 50°C for 48 h, and washed three times with 50 m*M* disodium EDTA for three days. The agarose blocks could then be stored in this solution for more than six months. Plugs about 3 mm long were cut from the agarose blocks and used for electrophoresis.

Pulse field gel electrophoresis The DNA preparations were loaded onto 1.0% (w/v) agarose gel (agarose H, Wako Pure Chem. Ind., Osaka) in electrophoresis buffer (45 m*M* Tris-base, 45 m*M* boric acid, 1.25 m*M* disodium EDTA, pH 8.3). Electrophoresis was carried out by using a CHEF apparatus (horizontal gel chamber, a model 200/20 power supply and Pulsewave 760 switcher; Japan Bio Rad Co.). The gels were run at 200 V at 10–15°C in electrophoresis buffer with a switching time of 60 s for 15 h followed by a switching time of 90 s for 8 h. DNA size standards prepared from yeast chromosomes (Japan Bio Rad Co.) were used as markers. Gels were stained in the dark with 1.5 μg/ml of ethidium bromide (Sigma Chemical Co.) overnight, destained in deionized water, and photographed on an ultraviolet light box with a Polaroid 667 positive-negative film.

RESULTS

Growth-inhibitory activity of DUMs and other typical antitumor agents In advance of the analysis of DNA fragmentation, the growth-inhibitory activity of DUMs against HeLa S_3 cells was compared with those of other typical antitumor agents, and the results are summarized in Table I. DUMA, DUMB1 and DUMB2 were about 1×10^3 times more potent than the other antitumor agents examined. The concentration of each compound to be used for further examinations was chosen on the basis of these IC_{50} values.

DNA fragmentation by DUMs To compare the effects of DUMs on cellular DNA, HeLa S_3 cells were treated with DUMs, and the extracted DNA was analyzed by agarose gel electrophoresis using CHEF apparatus. This method is sensitive enough to separate DNA as large as yeast chromosomal DNA (245–2200 kbp) (Fig. 2(A), lane 1). We applied this apparatus for the analysis of ultralarge DNA extracted from the drug-treated HeLa S_3 cells, and the results are summarized in Table II. The DNA of untreated cells was so large that it did not migrate in the running gel (1.0% agarose) under these conditions (Fig. 2(A), lane 2). However, DNA fragmentation became detectable in the cells treated with DUMs at 10 times their IC_{50} values shown in Table I. DUMA, DUMB1 and DUMB2 showed similar fragmentation patterns of DNA (Fig. 2(A), lanes 4 and 8; (B), lane 4, respectively), since DNA fragments of about 900, 1500 and 1800 kbp were detected in all three lanes. The DNA-fragmenting activ-

Table I. Growth-inhibitory Activity of Various Antitumor Agents against Human Uterine Cervix Carcinoma HeLa S_3 Cells

Group	Compound	IC_{50} (μM)
Duocarmycins	Duocarmycin A	0.00012
	Duocarmycin B_1	0.00042
	Duocarmycin B_2	0.00013
Intercalators	Actinomycin D	0.052
	Adriamycin	0.33
	Epirubicin	0.17
	Mitoxantrone	0.040
Radical producers	Bleomycin	11
	Neocarzinostatin	0.059[a]
Topoisomerase inhibitors	Camptothecin	0.32
	VP-16	2.2
	m-AMSA	0.086
Alkylating agents	Mitomycin C	1.0
	Cisplatin	6.4
Mitotic inhibitors	Vincristine	0.14
	Vinorelbine	0.82

a) Units/ml.

Table II. Summary of DNA Fragmentation by Antitumor Agents

Group	Compound	Concentration (ratio to IC_{50} value)		
		×1	×10	×100
Duocarmycins	Duocarmycin A	×[a]	○[b]	○
	Duocarmycin B_1	×	○	○
	Duocarmycin B_2	×	○	○
Intercalators	Actinomycin D	×	○	○
	Adriamycin	×	○	○
	Epirubicin	×	○	○
	Mitoxantrone	×	×	○
Radical producers	Bleomycin	○	○	○
	Neocarzinostatin	×	○	○
Topoisomerase inhibitors	Camptothecin	×	○	○
	VP-16	×	○	○
	m-AMSA	NT[c]	○	○
Alkylating agents	Mitomycin C	NT	×	×
	Cisplatin	×	×	×
Mitotic inhibitors	Vincristine	NT	×	×
	Vinorelbine	NT	×	×

a) DNA fragmentation was not detected.
b) DNA fragmentation was detected.
c) Not tested.

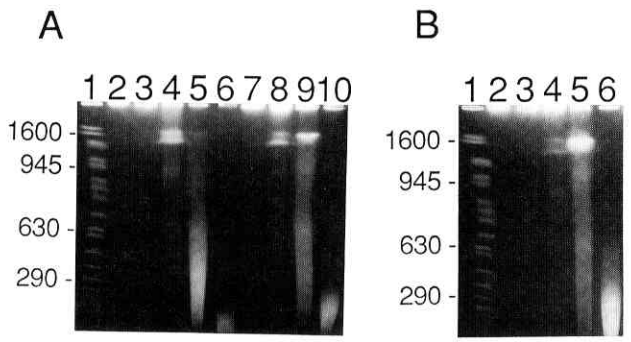

Fig. 2. Agarose gel electrophoresis of DNA extracted from DUMB1-, DUMB2- or DUMA-treated HeLa S_3 cells. HeLa S_3 cells precultured for 24 h were treated with DUMB1, DUMB2 or DUMA for 2 h. The DNA extraction and agarose gel electrophoresis with CHEF apparatus were performed as described in "Materials and Methods." Lanes of (A): 1, DNA size standard (kbp); 2, untreated; 3–6, DUMB1 0.42, 4.2, 42 and 420 nM; 7–10, DUMB2 0.13, 1.3, 13 and 130 nM. Lanes of (B): 1, DNA size standard (kbp); 2, untreated; 3–6, DUMA 0.12, 1.2, 12 and 120 nM.

ity of DUMs is unlikely to be associated with the cell death by nonspecific mechanisms, since the short treatment period of 2 h was enough to eliminate such mechanisms (data not shown). At 100 times their IC_{50} values, DNA was fragmented to smaller size and appeared as a smear ((A), lanes 5 and 9; (B), lane 5), indicating that the DNA-fragmenting activity of DUMs is concentration-dependent. These results also suggest that DUMA, DUMB1 and DUMB2 inhibited the growth of HeLa S_3 cells by the same mechanism.

DNA fragmentation by radical producers To elucidate the characteristics of activity of DUMs, DNA-fragmenting activities of other antitumor agents were examined and the results are summarized in Table II. First, DNA extracted from neocarzinostatin- or bleomycin-treated HeLa S_3 cells was analyzed (Fig. 3(A)), since these drugs were demonstrated to cleave DNA strands through the production of radicals in cell-free systems,[14, 15] and also in cellular DNA level by alkaline elution or alkaline sucrose density gradient centrifugation.[1, 3] The DNA fragmentation caused in neocarzinostatin- or bleomycin-treated mammalian cells was clearly detectable by electrophoresis using CHEF apparatus (Fig. 3(A)). Interestingly, the DNA fragmentation patterns caused by both drugs were rather similar to those of DUMs. DNA fragmentation by bleomycin occurred at 11 μM (lane 3), the IC_{50} value, suggesting that this activity of bleomycin is fundamentally associated with its growth-inhibitory activity. At higher concentrations, DNA was cleaved to fragments of 700 to 245 kbp (lane 4). DNA fragmentation patterns induced by neocarzinostatin were also similar to those by DUMs (lanes 6–8). However, DNA fragments of about 900 kbp, which were induced by DUMs, were not detected in bleomycin- or neocarzinostatin-treated cells.

DNA fragmentation by intercalators DNA fragmentation patterns of intercalator-treated cells were examined

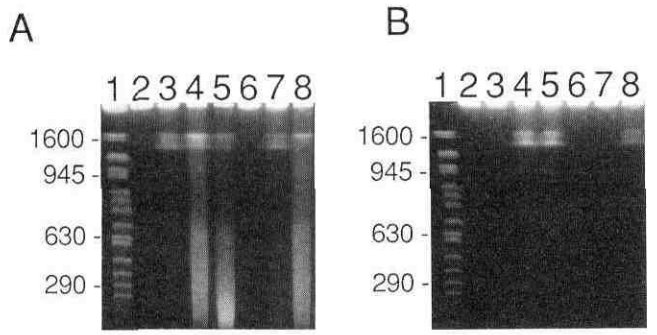

Fig. 3. Agarose gel electrophoresis of DNA extracted from radical producer- or intercalator-treated HeLa S_3 cells. Lanes of (A): 1, DNA size standard (kbp); 2, untreated; 3–5, bleomycin 11, 110 and 1100 μM; 6–8, neocarzinostatin 0.059, 0.59 and 5.9 units/ml. Lanes of (B): 1, DNA size standard (kbp); 2, untreated; 3–5, adriamycin 0.33, 3.3 and 33 μM; 6–8, mitoxantrone 0.040, 0.40 and 4.0 μM.

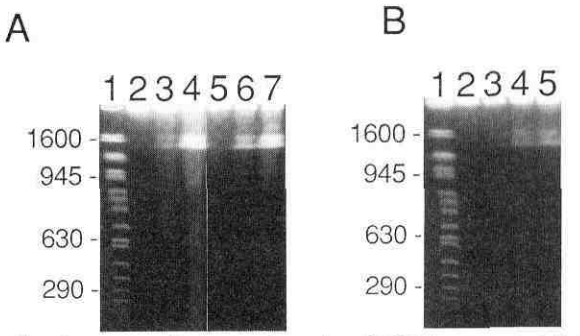

Fig. 4. Agarose gel electrophoresis of DNA extracted from topoisomerase inhibitor-treated HeLa S_3 cells. Lanes of (A): 1, DNA size standard (kbp); 2, untreated; 3, 4, m-AMSA 0.86 and 8.6 μM; 5–7, VP-16 2.2, 22 and 220 μM. Lanes of (B): 1, DNA size standard (kbp); 2, untreated; 3–5, camptothecin 0.32, 3.2 and 32 μM.

(Fig. 3(B)). Adriamycin or mitoxantrone induced DNA fragmentation in HeLa S_3 cells, although the patterns were different from those by DUMs. Adriamycin produced DNA fragments of about 1800 and 1500 kbp at 3.3 μM, 10 times its IC_{50} value (lane 4), and the same fragmentation pattern was observed at 33 μM (lane 5). In the case of mitoxantrone, DNA fragments of about 1800 and 1500 kbp were observed at 4.0 μM, 100 times its IC_{50} value (lane 8). Actinomycin D and epirubicin exhibited DNA fragmentation patterns similar to that of adriamycin (data not shown). DNA fragmentation was caused by these intercalators at higher concentrations than their IC_{50} values, and DNA fragments of less than 1200 kbp were scarcely observed.

DNA fragmentation by topoisomerase inhibitors Topoisomerase II inhibitors, m-AMSA and VP-16, were demonstrated to cleave cellular DNA strands by the alkaline elution method.[8,9] The DNA fragmentation caused in m-AMSA- or VP-16-treated mammalian cells was clearly detectable by electrophoresis using CHEF apparatus (Fig. 4 (A)). DNA fragmentation by m-AMSA occurred in the cells treated at 0.86 μM (lane 3), 10 times its IC_{50} value. DNA fragments of about 1800, 1500 and 900 kbp were observed in lane 3, and the intensity of these bands increased in a concentration-dependent manner (lane 4). Furthermore, broad bands of additional DNA fragments were generated around 600 kbp. In the case of VP-16, a similar pattern of DNA fragments was also observed (lanes 5–7), although DNA fragments below 900 kbp were not detectable. DNA fragmentation was also detected in the cells treated with camptothecin, a topoisomerase I inhibitor (Fig. 4(B)), and its pattern was similar to those induced by topoisomerase II inhibitors.

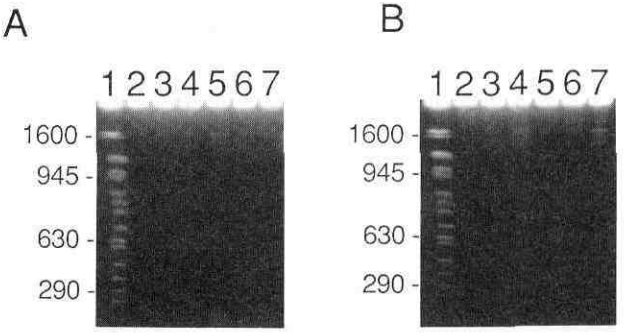

Fig. 5. Agarose gel electrophoresis of DNA extracted from DNA crosslinker- or mitotic inhibitor-treated HeLa S_3 cells. Lanes of (A): 1, DNA size standard (kbp); 2, untreated; 3–5, cisplatin 6.4, 64 and 640 μM; 6, 7, MMC 10 and 100 μM. Lanes of (B): 1, DNA size standard (kbp); 2, untreated; 3, 4, vinorelbine 8.2 and 82 μM; 5–7, vincristine 1.4, 14 and 140 μM.

DNA fragmentation by alkylating agents and mitotic inhibitors DNA-crosslinking agents, cisplatin and MMC, did not cause DNA fragmentation in HeLa S_3 cells at 10 times their IC_{50} values (Fig. 5(A), lanes 4 and 6), and only faint fragments at 100 times their IC_{50} values.

In the case of mitotic inhibitors such as vinorelbine and vincristine, DNA fragmentation was not detected by this method even at 100 times the IC_{50} values (Fig. 5(B), lanes 4 and 6).

DISCUSSION

The fragmentation of DNA in mammalian cells treated with antitumor agents has been examined by the alkaline elution method or alkaline sucrose density gradi-

ent method.[1-6] However, these methods need radioactive materials as well as technical skill, and they are inappropriate for handling many DNA samples simultaneously. Furthermore, the alkaline elution method provides no information on the size of the DNA fragments, although the intensity of DNA fragmentation can be measured. The advantages of the CHEF method used in this report are as follows. Without any radioactive materials, DNA fragmentation can be detected. This technic is sensitive enough to separate huge DNA fragments of about 1600 kbp. The patterns of DNA fragmentation can be compared visually as if they were "fingerprints." Furthermore, the same DNA sample can be analyzed repeatedly to confirm the reproducibility or for comparison with other DNA samples, since DNA samples handled as agarose plugs were stable at 4°C for at least six months.[8]

Although the activities of intercalators, radical producers and topoisomerase inhibitors have already been demonstrated by alkaline elution or alkaline sucrose density gradient methods,[1-3,5,6] the results of agarose gel electrophoresis provide new indications of the size of DNA fragments (Figs. 3 and 4). Namely, two bands of DNA fragments of about 1800 and 1500 kbp were commonly detected in the cells treated with these antitumor agents. In addition, DNA fragments of about 900 kbp were detected in the cells treated with topoisomerase inhibitors, m-AMSA, VP-16 or camptothecin, and those with a broad size range between 700 and 245 kbp in cells treated with radical producers, bleomycin and neocarzinostatin. Thus this method provides "fingerprints" of the DNA-fragmenting activity of various antitumor agents.

The electrophilic cyclopropane present in DUMA (Fig. 1) was demonstrated to induce the N3 alkylation of adenine of DNA strands.[16,17] The fact that DUMA has the most potent growth-inhibitory activity among DUMs may be attributed to its cyclopropane structure. The halogens of DUMB1 and DUMB2 are supposed to be lost, resulting in the formation of DUMA as a terminal active compound.[16] The similar patterns of DNA fragmentation detected in the cells treated with DUMB1, DUMB2 and DUMA (Fig. 2) may support the activation of DUMB1 and DUMB2 to DUMA. The DNA fragmentation pattern induced by DUMs was characteristic, since the types of fragments induced by topoisomerase inhibitors and radical producers were simultaneously detected, suggesting a novel mechanism of interaction of DUMs with DNA.

Typical DNA fragments of about 1800, 1500, 900 and 600 kbp were detected after treatment with various antitumor agents (Figs. 3 and 4). These results may be associated with the structure of DNA packed in the chromatin. Jan Filipski *et al.* reported the periodicity of DNA folding in higher order chromatin structures.[9] The considerable literature on chromatin structure[18,19] suggests that there are five levels of folding of the DNA as nucleosomes, solenoids, loop, rosette and coil. At the third level, the 30 nm fiber organized from nucleosomes is folded to form a loop which is composed of 50 kbp of DNA. These DNA loops are folded into hexameric rosettes containing 300 kbp of DNA and form a fiber of 200–300 nm in diameter. The periodical difference of the size of DNA fragments, 300 kbp, observed in our experiments may suggest the existence of a hypersensitive site to the above antitumor agents in a rosette. Further investigations are planned in this regard.

As for the biological significance of DNA fragmentations by antitumor agents, the cell growth-inhibitory activity of bleomycin is indicated to be actually due to its DNA-fragmenting activity, since this activity was detected at the IC_{50} value (Table II). DUMs, intercalators, neocarzinostatin and topoisomerase inhibitors did not induce DNA fragmentations until 10 times their IC_{50} values (Table II). However the fragmentation pattern of these compounds correlated well with their DNA-damaging actions reported in other systems.[2] We suppose that the DNA fragmentations induced by these agents at 10 times their IC_{50} values exceed the minimum necessary to induce the 50% growth inhibition of HeLa S_3 cells. DNA damage at the IC_{50} values, which was faint and not detectable by pulse field gel electrophoresis, may be enough for minimum cell growth inhibition. The cell growth-inhibitory activity of these agents is also considered to be due to their DNA-fragmenting activities, with the exception of camptothecin.

Camptothecin, a topoisomerase I inhibitor, was not expected to induce the fragmentation of double-stranded DNA since it induces the cleavage of only one strand of DNA. However in our experiment, DNA fragmentation was detected with CHEF apparatus (Fig. 4(B)). The same phenomenon had already been reported in human leukemia HL-60 cells[20] and SV40MRC5 cells,[21] suggesting that camptothecin possesses a distinctive action mechanism at the cellular DNA level.

Cisplatin and MMC did not cause DNA fragmentation even at 100 times their IC_{50} values (Fig. 5(A)). These results seem reasonable, since they are reported to form DNA-interstrand crosslinks,[22,23] which might not be detected as the DNA fragmentation by CHEF apparatus. We are now examining a modified method to detect the activity of these DNA-crosslinkers using CHEF apparatus. The results for mitotic inhibitors, vinorelbine or vincristine, are also reasonable (Fig. 5(B)).

In conclusion, pulse field gel electrophoresis should be useful for characterizing the DNA-cleaving activity of various antitumor agents at the cellular level. DUMs were confirmed to possess strong and characteristic DNA fragmentation activity by this method.

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