Differential Induction of Chromosomal Aberrations by Topoisomerase Inhibitors in Cultured Chinese Hamster Cells

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The inducibility of chromosomal aberration was studied using five mammalian topoisomerase inhibitors in Chinese hamster lung fibroblastic cells (CHL) in two protocols involving 6 and 24 h treatment, with respect to the mechanism of chromosomal aberration. In the 6 h treatment plus 18 h recovery culture (6 h treatment), a high incidence of chromosome-type aberration was observed with three mammalian topoisomerase II (topo-II) inhibitors: etoposide, adriamycin and mitoxantrone, which stabilize the cleavable complex of topo-II and DNA. In the 24 h continuous treatment (24 h treatment), these chemicals induced mainly chromatid-type aberrations. The chromosome-type aberrations which appeared as a result of the 6 h treatment were primarily induced, because the majority of aberrant cells induced by the inhibitors remained in the first metaphase, as determined by the 5-bromodeoxyuridine labelling method. This also indicates that the cells were sensitive to these topo-II inhibitors at the G₁-S boundary phase. Aclarubicin, however, which does not form the cleavable complex but is an antagonist against topo-II, and camptothecin, an inhibitor of topoisomerase I, mainly induced chromatid-type aberrations, irrespective of the duration of treatment. These results suggest that the differential induction of chromosomal aberrations by topoisomerase inhibitors reflects different mechanisms of inhibition by the topoisomerases, and that DNA double strand breakages promoted by the stabilization of the cleavable complex formed by topo-II are closely associated with the induction of chromosome-type aberration.

Keywords chromosomal aberration; topoisomerase inhibitor; action mechanism

Structural chromosome alterations are generally characterized as chromatid-type and chromosome-type aberrations. DNA damage which occurs either before or after DNA replication produces chromatid-type or chromosome-type aberrations, respectively. Most clastogenic chemicals are known to produce mainly chromatid-type aberrations in mammalian cells, regardless of their cell cycle stage. They are called S-dependent agents, since the induction of chromatid-type aberrations requires DNA lesions to pass through DNA synthesis.¹⁾ Ionizing radiation, such as X- and γ -rays, the so-called S-independent agents, induce chromosome-type aberrations when cells are irradiated in the G₁ phase of the cell cycle (before DNA synthesis), or chromatid-type aberrations in the G₂ phase (after DNA synthesis). These agents also induce both types of aberrations, but predominantly chromatid-type aberrations, in the S phase (*i.e.*, the DNA synthesis period), respectively.²⁾ Bleomycin and restriction endonucleases are also known to be S-independent agents.^{3,4)} Additionally, chromosome-type aberrations can be caused by certain kinds of intercalators, *e.g.* daunomycin.⁵⁾ However, there have been few reports on preferential induction by chemicals.

DNA topoisomerases regulate the topology of DNA in the processes of replication, transcription, recombination and repair.⁶⁾ The enzymes are classified into two types, with different catalytic activities for relaxing torsioned DNA strands on chromatin: type I topoisomerase (topo-I) for ATP-independent breaking of DNA single strands of chromatin; and type-II topoisomerase (topo-II) for ATP-dependent breaking of DNA double strands of chromatin. The inhibition of topo-I or topo-II causes an accumulation of cleavable complexes of DNA and the enzyme with covalent binding, followed by respective breakage of single or double strands of DNA.⁶⁾ Recently, several types of

inhibitors of bacterial or mammalian topo-I, bacterial topo-II (gyrase) and eukaryotic topo-II have been identified. Eukaryotic topo-II inhibitors such as 4'-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA), 4'-demethylepipodophyllotoxin-9-(4,6-*O*-2-ethenylidene- β -D-glucopyranoside) (etoposide, VP-16), and aclarubicin are used in cancer chemotherapy,⁷⁻⁹⁾ although the mechanism of their selective cytotoxicity is not clear. Among these, *m*-AMSA has been shown to induce chromosome-type aberrations in human lymphocytes.^{10,11)} From this finding, it can be suggested that topoisomerase inhibitors contribute to the induction of chromatid-type or chromosome-type aberrations due to the accumulation of cleavable topoisomerase-DNA complexes, thereby presumably giving an insight into the mechanism of drug-induced chromosomal aberration, or of their selective toxicity to malignant cells. However, the role of topoisomerase inhibitors on the induction of chromosomal aberration has not yet been studied.

Thus, the present study was undertaken to characterize chromosomal aberrations induced by various kinds of eukaryotic topoisomerase inhibitors in cultured Chinese hamster cells, and shows the frequent induction of chromosome-type aberrations in cells treated with topo-II inhibitors.

MATERIALS AND METHODS

Chemicals Four topo-II inhibitors (below) and one topo-I inhibitor (camptothecin) were used: adriamycin and aclarubicin hydrochloride were purchased from Wako Pure Chemical Co., Osaka (Japan); camptothecin from Sigma Chemical Co., (U.S.A.); etoposide from Nippon Kayaku Co., Ltd. (Japan); and mitoxantrone hydrochloride from Takeda Chemical Industries, Ltd. (Japan).

Cell Culture Chinese hamster lung fibroblastic cells (CHL) established by Koyama *et al.*¹²⁾ were cultured in Eagle's minimum essential medium supplemented with 10% calf serum under 5% CO₂-air for 4 d.

For analysis of chromosomal aberrations in CHL cells, two protocols which differed in the duration of chemical treatment (6 or 24 h treatment) were used, taking into consideration that the doubling time of CHL cells was 15–17 h.¹³⁾ The chemical doses used in the chromosomal aberration test were higher for the 6 h treatment than for the 24 h treatment, because of higher cell toxicity in the longer treatment, as observed in preliminary experiments. The chemical treatment of CHL cells was as follows: for the 6 h treatment, each chemical was added to the cultures on the 3rd day of culture. After 6 h incubation with the chemical, the cells were washed once with phosphate buffered saline, followed by a further 18 h of incubation in fresh medium without the chemical. For the 24 h treatment, each chemical was added into the culture on the 3rd day and was incubated for 24 h. Then, the chromosome preparations of each culture were prepared to be assayed for chromosomal aberrations. The characteristics of the types of chromosomal aberrations were confirmed by repeated experiments. In each experiment, two culture dishes were used for each dose of each chemical. One dish was used to prepare the chromosomal specimens, and another dish was used to confirm the cell cycle number by determining sister chromatid exchanges (SCE).¹⁴⁾

Chromosome Preparation In order to accumulate cells at the metaphase, colcemid (0.2 µg/ml) was added to each culture at 2 h before harvesting the cells. For the SCE tests, 5 µM bromodeoxyuridine (BrdU) was added to the cells under dark conditions 22 h before the addition of colcemid. At the end of incubation, the cells were trypsinized and centrifuged at 1000 rpm. The cell pellets were resuspended in a hypotonic solution of 0.075 M KCl and incubated for 15 min at 37 °C. Then the cells were centrifuged and fixed with methanol–acetic acid (3:1 by volume) at room temperature for 10 min. The fixation process was repeated three times. Specimens for chromosomal analysis were prepared by the air-dry method.¹⁵⁾ The cell cycle number of the aberrant cells was determined by the staining of SCE using the fluorescent and Giemsa methods.¹⁶⁾

One hundred cells at the metaphase on each specimen were analyzed for chromosomal aberrations and SCE. Structural aberrations were classified as chromatid gap, including chromosome gap (gap), chromatid break (ctb), chromatid exchange (cte), chromosome break (csb), chromosome exchange (cse; further divided into dicentric and ring chromosomes), fragmentation (fra), and cells with multiple aberrations (mul). Every cell with any type of aberration in the metaphase was recorded as an aberrant cell. If different types of aberrations were observed in a cell, the frequency of the individual aberration type was recorded as one. Results were expressed as the mean of two specimens on each culture. The frequency of aberrant cells was calculated, excluding cells having only gaps. Positive clastogenicity was judged for an aberrant frequency above 10% with dose dependency.¹⁷⁾ The fre-

quency of chromosome exchanges in the untreated cells was less than 0.08%.¹⁸⁾ Accordingly, in the case of chromosome-type aberrations (especially chromosome exchanges), frequencies above approximately 10% of the observed cells were considered as showing a high incidence of aberration.

RESULTS

Analysis of Chromosomal Aberrations in Cells Treated with Topoisomerase Inhibitors The typical results of the chromosomal aberration tests are shown in Table I. In all cases treated with chemicals, a few chromosomal gaps appeared, including only chromatid-types. In the 6 h treatment, all topoisomerase inhibitors produced frequent chromatid-type aberrations (ctb, cte). It is notable, however, that etoposide, adriamycin and mitoxantrone, which are topo-II inhibitors, caused high frequencies of chromosome-type aberrations (csb, cse), dose dependently. These three topo-II inhibitors showed chromosome exchange frequencies of above 10%. There was no significant difference between the frequencies of dicentric and ring chromosomes in the chromosome exchanges. The frequency of chromosome breaks in the aberrant cells was from 52.1 to 63.3% for etoposide, from 39.6 to 61.5% for adriamycin, and from 23.5 to 27.8% for mitoxantrone, respectively. The frequency of chromosome exchange in the aberrant cells was from 29.6 to 38.9% for etoposide, from 14.6 to 28.2% for adriamycin, and from 17.6 to 27.8% for mitoxantrone, respectively. In contrast, chromosome-type aberrations were less frequently induced by aclarubicin or camptothecin treatment. The frequency of chromosome exchange in the observed cells was less than 10% for both chemicals. The frequency of chromosome breaks in the aberrant cells was slightly higher at lower doses of both chemicals, but the number of aberrant cells with chromosome breaks was negligible.

In the 24 h treatment, each chemical induced the production of mainly chromatid-type aberrations (chromatid break and exchange). The frequencies of chromosome-type aberrations following etoposide, adriamycin and mitoxantrone treatments were markedly reduced in comparison to those of the 6 h treatment, although the frequency of chromosome breaks was still relatively high (etoposide; 1–14%, adriamycin; 2–8%, mitoxantrone; 2–12%). The frequency of chromosome breaks following aclarubicin or camptothecin treatment were 1% or 2–7%, respectively. The frequency of chromosome exchange was less than 10% in all cases.

The Sister Chromatid Exchange Analysis of Cells Treated with Topoisomerase Inhibitors In Table II, the results of the SCE analysis are expressed as ratios of the first and second metaphases in normal or aberrant cells at 18 h after 6 h treatment of the inhibitors. Solvent control (10% saline by volume in the medium) of the etoposide treatment gave values of 21% on the first metaphase (M1) and 79% on the second metaphase (M2). The etoposide treatment delayed the cell cycle of normal cells dose dependently: it increased the first metaphase to 88%, and decreased the second metaphase to 12%. Moreover, most aberrant cells induced by topoisomerase inhibitors were

TABLE I. Chromosomal Aberrations Induced by 6 or 24 h of Treatment with Various Topoisomerase Inhibitors in Chinese Hamster Cells

Treatment time (h) ^{a)}	Dose ($\mu\text{g/ml}$)	Frequencies of structural aberrations (n) ^{b)}							Aberrant cells (%)
		gap	ctb	cte	csb	cse	fra	mul	
Etoposide 6-18	0	1	0	0	0	0 (0, 0) ^{c)}	0	0	0
	0.6	1	3	11	19	11 (5, 7)	0	0	30
	0.8	1	0	15	30	21 (13, 8)	0	6	54
	1.0	1	3	24	37	21 (11, 12)	0	11	71
	24-0	0	2	0	0	0 (0, 0)	0	0	2
24-0	0.075	5	1	4	1	0 (0, 0)	0	0	6
	0.15	3	10	15	7	2 (2, 0)	0	0	28
	0.30	2	12	22	14	6 (3, 3)	0	0	70
Adriamycin 6-18	0	0	1	0	0	0 (0, 0)	0	0	1
	0.10	1	3	18	24	11 (3, 8)	0	0	39
	0.15	0	2	42	41	21 (12, 11)	2	19	86
	0.20	1	0	57	38	14 (6, 10)	6	22	96
	24-0	0	0	0	0	0 (0, 0)	0	0	0
24-0	0.013	0	4	8	0	0 (0, 0)	0	0	11
	0.025	3	12	25	2	0 (0, 0)	0	0	34
	0.05	1	23	70	8	1 (0, 1)	0	2	79
Mitoxantrone 6-18	0	1	1	0	0	0 (0, 0)	0	0	1
	0.01	6	3	11	5	4 (1, 3)	0	0	18
	0.02	2	5	21	10	10 (6, 4)	0	0	36
	0.03	3	15	63	20	15 (8, 7)	0	7	85
	24-0	0	0	0	0	0 (0, 0)	0	0	0
24-0	0.005	3	8	14	2	1 (1, 0)	0	0	24
	0.010	1	19	58	7	2 (0, 2)	0	0	67
	0.015	4	41	73	12	4 (0, 4)	1	13	93
Aclarubicin 6-18	0	1	1	0	0	0 (0, 0)	0	0	1
	0.3	1	4	19	2	0 (0, 0)	0	0	23
	0.4	1	9	25	1	1 (0, 1)	0	0	32
	0.6	5	10	42	3	1 (0, 1)	0	7	64
	24-0	0	0	0	0	0 (0, 0)	0	0	0
24-0	0.125	1	1	8	0	0 (0, 0)	0	0	9
	0.188	0	4	14	1	1 (0, 1)	0	0	19
	0.250	4	24	76	1	1 (0, 1)	0	4	85
Camptothecin 6-18	0	0	0	0	0	0 (0, 0)	0	0	0
	0.075	0	5	11	2	2 (2, 0)	0	2	18
	0.10	1	9	28	6	1 (0, 1)	0	7	39
	0.15	0	14	38	5	0 (0, 0)	2	14	57
	24-0	0	1	0	0	0 (0, 0)	0	0	1
24-0	0.015	0	5	15	0	0 (0, 0)	0	0	19
	0.03	1	8	41	2	1 (1, 0)	0	0	48
	0.06	3	25	63	7	1 (0, 1)	0	30	97

The frequency of structural aberration is shown as the mean of two chromosome preparations from each culture. One hundred cells were analyzed for each preparation. *a)* Treatment time-recovery time. *b)* n , Number of cells with aberration types as follows: gap, chromatid gaps (including chromosome gaps); ctb, chromatid breaks; cte, chromatid exchanges; csb, chromosome breaks; cse, chromosome exchanges; fra, fragmentation; mul, cells with multiple aberrations. *c)* Numbers in parentheses represent the frequencies of dicentric and ring chromosomes, respectively.

counted as the first metaphase, except for cells treated with mitoxantrone at a dose of 0.01 $\mu\text{g/ml}$. This indicates that most aberrant cells induced by etoposide, adriamycin and mitoxantrone remained in the first cell cycle and did not proceed to the second cell cycle.

DISCUSSION

In the present study, five topoisomerase inhibitors showed different abilities to induce chromosomal aberrations under varying durations of treatment. Chromatid-type aberrations were induced mainly by 24 h treatment with each inhibitor, with a comparatively high incidence

of induction following 6 h of treatment. These inductions are considered to be related to biological events such as the inhibition of DNA and RNA polymerases, the production of active oxygen species, and misrepair.¹⁹⁻²³⁾ The distinguishing characteristic of chromosomal aberrations induced by the 6 h treatment in this system was the frequent induction of chromosome-type aberrations, especially that of chromosome exchanges above 10%. Namely, three topo-II inhibitors with stabilization of the cleavable complex,^{6,24)} etoposide, adriamycin and mitoxantrone, showed high inducibility of chromosome-type aberrations (chromosome break and exchange), in contrast to less inducibility of the chromatid-type aberrations. On

TABLE II. Ratios of the First Metaphase (M1) and the Second Metaphase (M2) in Chinese Hamster Cells Treated with Topoisomerase Inhibitors for 6 h

Chemical	Dose ($\mu\text{g/ml}$)	% in normal cells		% in aberrant cells	
		M1	M2	M1	M2
Etoposide	0	21	79	n.t. ^{a)}	
	0.6	58	42	97	3
	0.8	77	23	99	1
	1.0	88	12	100	0
Adriamycin	0			n.t.	
	0.1	n.t.		100	0
	0.15			100	0
	0.2			100	0
Mitoxantrone	0			n.t.	
	0.01	n.t.		80	20
	0.02			99	1
	0.03			100	0
Aclarubicin	0			n.t.	
	0.3	n.t.		100	0
	0.4			100	0
	0.6			100	0
Camptothecin	0			n.t.	
	0.075	n.t.		100	0
	0.10			100	0
	0.15			100	0

One hundred cells were analyzed for each preparation. a) Not tested.

the other hand, aclarubicin, a eukaryotic topo-II inhibitor without a stabilizing cleavable complex,²⁵⁾ induced mainly chromatid-type aberrations. It is possible that the chromosome-type aberrations induced during the 6 h treatment include the derived-type aberrations which could be formed if cells with chromatid-type aberrations passed through the second cell cycle.²⁾ However, the SCE analysis (Table II) showed that most aberrant cells remained in the first cell cycle. It was therefore confirmed that the observed chromosome-type aberrations were not derived from chromatid-type aberrations, but were primarily induced by topoisomerase inhibitors. However, as an exception, since 20% of the aberrant cells at 0.01 $\mu\text{g/ml}$ of mitoxantrone were observed at the second metaphase, their aberrations may partly include derived-type aberrations.

It has been considered that the induction of chromosomal aberrations is caused by DNA breakage.²⁶⁾ The mechanism of DNA breakage are proposed to be as follows²⁷⁾: 1) direct scission by agents such as X-ray, H_2O_2 and oxygen radicals; 2) hydrolysis of the sugar-phosphate backbone at sites modified by chemicals which damage or bind covalently to nucleotide sugars and bases; and 3) enzymatic scission by topoisomerases, endonucleases and lysosomal DNA hydrolases. High frequencies of chromosome-type aberrations observed in the 6 h treatment with etoposide, adriamycin or mitoxantrone may be due to topo-II inhibition by the stabilization of a cleavable complex, considering that camptothecin, a eukaryotic topo-I inhibitor, induced mainly chromatid-type aberrations. This result is similar to the aberration on the induction of chromatid-type aberrations in human lymphocytes treated with camptothecin at the G_1 phase.²⁸⁾ Moreover, in 6 h of treatment with aclarubicin, which

does not stabilize the cleavable complex, the induction of chromosome-type aberrations was hardly observed. This is further supported by similar observations of two eukaryotic topo-II inhibitors, *m*-AMSA and 5-imino-daunorubicin, which also stabilize the cleavable complex.^{10,22,29)}

The 6 h duration of treatment occupies about one third of the cell cycle of CHL cells. During the 6 h treatment, most of the CHL cells, even unsynchronized ones, may progress to the G_1 -S phase. It was reported that the topo-II contents began to increase with the initiation of DNA replication, and continued to increase through the S- G_2 phase.³⁰⁾ On the other hand, in the present study, the doses of the 6 h treatment required to induce chromosome exchanges of about 10% were 0.6–1 $\mu\text{g/ml}$ in etoposide, 0.1–0.2 $\mu\text{g/ml}$ in adriamycin and 0.02–0.03 $\mu\text{g/ml}$ in mitoxantrone. Correspondingly, the doses of these chemicals required to produce the cleavable complex detected by the topoisomerase inhibition assay were 7.5 $\mu\text{g/ml}$ in etoposide, 0.01–0.25 $\mu\text{g/ml}$ in adriamycin and 0.05–0.25 $\mu\text{g/ml}$ in mitoxantrone, for 30 min or 1 h of treatment.^{24,31)} There is a similarity between the effective doses of the three topo-II inhibitors in the chromosomal aberration tests and the topoisomerase inhibition assays. Accordingly, it is considered that these three chemicals can act in the G_1 -S boundary phase and inhibit topo-II. So, if a chemical causes DNA double strand breakage by the inhibition of topo-II activity in the G_1 -S boundary phase, chromosome-type aberrations can be induced in the cell. This may be one of the reasons for the frequent induction of chromosome-type aberrations by 6 h treatment with the three topo-II inhibitors. Moreover, topo-II inhibitors such as etoposide and *m*-AMSA are considered to display cellular effects similar to those of X-rays.³²⁾ Ionizing radiation is required at about 10-fold intensity from double strand breaks in comparison with single strand breaks.³³⁾ In this study, the doses of etoposide and adriamycin used differed several-fold between the 6 h and 24 h treatments. So, like ionizing radiation, when the cells are treated with higher doses in the 6 h treatment, they may show higher frequencies of double strand breaks resulting from the formation of the cleavable complex. This may be another reason for the high frequencies of chromosome-type aberrations as a result of the 6 h treatment. It is reported that chemically-induced cleavable complexes are responsible for cytotoxicity.³⁴⁾ So, the fact that very few instances of chromosome exchange appear during the 24 h treatment may be related to rapid cell death of cells which have formed a cleavable complex.⁶⁾

Our results on the preferential inducibility of chromosome-type aberrations indicate that the induction of chromosome-type aberrations is closely associated with topo-II inhibition due to the stabilization of the cleavable complex. Moreover, this suggests that the CHL chromosomal aberration assay system of the 6 h treatment is a simple and useful way to estimate the inducibility of chromosome-type aberrations.

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