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Inhibitory Effects of Sizofiran on Anticancer Agent- or X-Ray-induced Sister Chromatid Exchanges and Mitotic Block in Murine Bone Marrow Cells

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The inhibitory effects of a biological response modifier (BRM), sizofiran, on sister chromatid exchanges (SCEs) in the bone marrow cells of mice treated with various anticancer agents or irradiation were investigated. Sizofiran (10 mg/kg i.m.) inhibited SCEs induced by mitomycin C (2 mg/kg i.v.), adriamycin (20 mg/kg i.v.) and cyclophosphamide (20 mg/kg i.v.) by about 20%, respectively. Analysis of the SCEs *in vivo* after irradiation plus sizofiran indicated that SCE levels were significantly lower than those observed in mice exposed to irradiation without sizofiran. Moreover, the effects of sizofiran were dependent on the timing of administration. Our results indicated that sizofiran should be administered simultaneously or soon after irradiation in order to minimize damage. Sizofiran also markedly restored the bone marrow cell mitosis which had been suppressed by anticancer agents, and this action was closely correlated with the prevention of increase in SCEs. These results indicate that in addition to immunopotentiating activity, sizofiran may play a role in preventing chromosomal damage induced by cancer chemotherapy and radiotherapy.

Key words: Sister chromatid exchange — Sizofiran — Irradiation — Anticancer agent

A number of widely used anticancer agents and radiation are known to have mutagenic potential toward bacteria and mammalian cells *in vitro* and *in vivo*.[1-4] Because of this mutagenic potential, these agents may induce heterogeneity in tumor cells to enhance tumor progression.[5] It has recently been pointed out that mutations caused by chemotherapy or rediotherapy may lead to the induction of secondary neoplasms.[6] Although the sister chromatid exchange (SCE) assay has been utilized for identifying genotoxic agents,[7,8] and for testing chemosensitivity,[9,10] little is known about the relationship between the frequencies of SCE and bone marrow toxicity induced by chemotherapy or radiotherapy. On the other hand, it has been reported that many immunopotentiators are beneficial in cancer treatment when combined with chemotherapy or radiotherapy.[11,12] Besides the immune-enhancing activity, other actions of the immunopotentiators may be beneficial for the therapy; for example, immunopotentiating agents may inhibit SCEs induced by chemotherapy or radiotherapy.[13]

Sizofiran, a 1,3 β-glucan[14] having the molecular structure shown in Fig. 1, displays anticancer activities by enhacing host immune responses, such as increasing the production of interleukin-2 and -3 by mitogen-stimulated spleen cells,[15-17] activating macrophages[18] and alleviating bone marrow cell damage induced by anticancer agents.[19]

In the present study, we investigated SCE frequencies and mitotic indices in bone marrow cells obtained from mice treated with anticancer agents or irradiation, and whether or not sizofiran affects such treatment-induced damage. The results indicate that sizofiran markedly restores mitotic indices suppressed by anticancer agents or irradiation and simultaneously prevents increases in the frequencies of SCE. The possible significance of sizofiran therapy in cancer patients treated with chemotherapy or radiotherapy is discussed.

MATERIALS AND METHODS

Animals Male C3H/He mice were purchased from Nippon SLC Co., Ltd. (Shizuoka). All mice were 7 weeks old and weighed 24 ± 2 g. The animals were kept in specific-pathogen-free animal rooms.

SCE analysis 5-Bromo-2′-deoxyuridine tablets (BrdUrd; Boehringer Mannheim Biochemicals, Indianapolis, IN) were inserted under the dorsal skin of C3H/He mice at a dose of 50 mg/kg. Twenty-one hours after the treatment, the animals were intraperitoneally administered 4 mg/kg of colchicine (Nacalai Tesque, Inc., Kyoto) and killed by cervical dislocation 3 h later. The femurs and tibias were removed, and stripped of the adherent muscles. Each bone was cleaned with 70% ethyl alcohol, and the epiphyses of the bones were cut off with scissors. The bone marrow cells were flushed out with physiological saline into a tube and centrifuged at 1500 rpm for 5 min. The supernatant was removed, and the pellet was resus-

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pended in a 0.075 *M* potassium chloride solution for 15 min at 37°C then recentrifuged. The cells were fixed for 10 min with 3 changes of a methanol:acetic acid (3:1, v/v) fixative. The cells were then resuspended in approximately 0.5 ml of fixative, dropped onto clean, chilled, wet slides and air-dried for 1 week at room temperature. Staining for SCE analysis was carried out according to a modified version of the technique developed by Perry and Wolff.[20] Slides were stained for 10 min with 10 μg/ml of Hoechst 33258 (Bisbenzimide H 33258, Nacalai Tesque, Inc.), rinsed briefly in tap water, and mounted in phosphate buffer (pH 7.0) with a cover slip. The slides were exposed to a 100 W electric light at a distance of 20 cm for 1 h. The coverslips were removed by rinsing with tap water, and the slides were incubated in 1 *M* NaH_2PO_4 (pH 8.0, 80°C) for 10 min, rinsed, and stained in 4% Giemsa-1/15 *M* phosphate buffer, pH 6.4 (Iatron Laboratories, Inc., Tokyo) for 10 min. All slides were coded and cells with 40 chromosomes were analyzed for SCEs. For statistical evaluation, the significance of differences was evaluated using Student's *t*-test compared to the respective control values. Percentage inhibition was calculated using the following formula:

$$100 - \frac{\text{number of SCEs/cell in the presence of sizofiran}}{\text{number of SCEs/cell in the absence of sizofiran}} \times 100.$$

The number of spontaneous SCEs per cell was subtracted from the numerator and the denominator.

Induction of *in vivo* SCE Mitomycin C, adriamycin (MMC, ADM; Kyowa Hakko Ltd., Tokyo) and cyclophosphamide (CY; Shionogi Ltd., Osaka) were dissolved in sterile phosphate-buffered saline (PBS) for SCE-induction and intravenously administered at different doses in 0.2 ml of PBS, 1 h after the implantation of BrdUrd. Sizofiran (Kaken Pharmaceutical Co. Ltd., Tokyo) was dissolved in PBS and given intramuscularly to animals immediately after the anticancer agent treatment. The control group received an equivalent volume of PBS. In *in vivo* SCE-induction studies with irradiation, mice were placed in a compartmentalized plastic restrainer and exposed to varying doses of whole-body irradiation from an MBR-1520R Biological X-irradiator (Hitachi Instrument Co., Tokyo) with a 0.5 Al-0.1 Cu filter, under a tube voltage of 150 kV and a tube current of 20 mA. The BrdUrd implantation into the animals was commenced 24 h before they were killed.

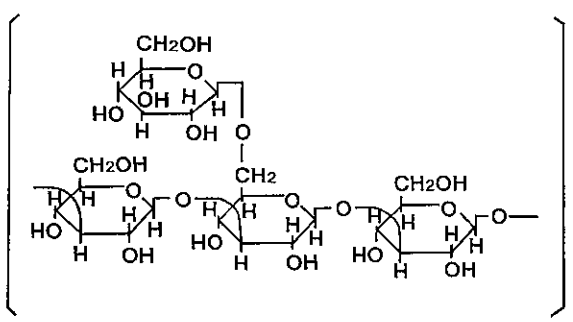

Fig. 1. Molecular structure of sizofiran. Chemical name: poly [3→[O-β-D-glucopyranosyl-(1→3)-O-[β-D-glucopyranosyl-(1→6)]-O-β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl]→1], molecular weight=450 kD.

RESULTS

Effect of sizofiran on SCE and mitotic index of bone marrow cells in mice treated with anticancer agents The results of the *in vivo* SCE and mitotic index studies in bone marrow cells of mice following exposure to anticancer agents are shown in Table I. The data were obtained from 4 animals in each group. The number of SCEs in mice treated with anticancer agents significantly increased as compared to PBS-treated controls, the frequencies being dose-dependent. On the other hand, the bone marrow cell mitotic index in mice treated with the anticancer agents decreased. As shown in Fig. 2, the number of SCEs in mice treated simultaneously with the agents and sizofiran was markedly reduced and the mitotic index was enhanced as compared to mice treated with the agents alone (Fig. 3).

Effect of sizofiran on SCE and mitotic index of bone marrow cells in mice treated with irradiation Changes in SCEs and mitotic indices after irradiation *in vivo* indi-

Table I. Induction of SCEs by Anticancer Agents in Bone Marrow Cells

Treated with (mg/kg)		SCEs/cell (mean±SD)	Mitotic index (%)
None		3.4±0.4	5.1
MMC	2.0	29.5±5.4*	1.7
	1.0	16.5±3.1*	2.8
	0.5	14.8±3.0*	5.2
CY	40.0	22.5±3.4*	4.2
	20.0	21.5±3.1*	4.8
	10.0	13.0±2.2*	5.6
ADM	20.0	17.8±2.6*	3.3
	10.0	15.0±4.1*	3.7
	5.0	8.8±1.7*	4.1

Four animals were used in each treatment group and 25 second-division metaphases were scored for SCEs in bone marrow cells from each animal. Anticancer agents were administered i.v. at the indicated dose.

* $P<0.05$ as compared with the untreated mice and calculated by using Student's *t* test.

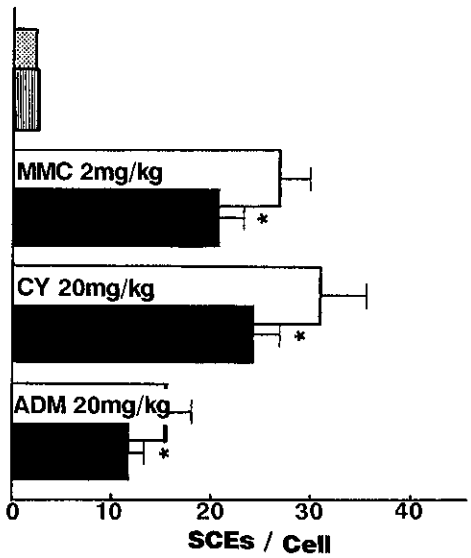

Fig. 2. Effect of sizofiran on anticancer agent-induced SCEs in mouse bone marrow cell. Four animals were used in each treatment group and 25 second-division metaphases from each animal were scored for SCEs. Anticancer agents and sizofiran were administered i.v. and i.m. respectively at the indicated doses. ▨ PBS control; ▥ sizofiran (SPG; 10 mg/kg) alone; □ anticancer agent alone; ■ anticancer agent combined with sizofiran. * $P<0.05$ as compared with anticancer agent-treated mice calculated by using Student's t test.

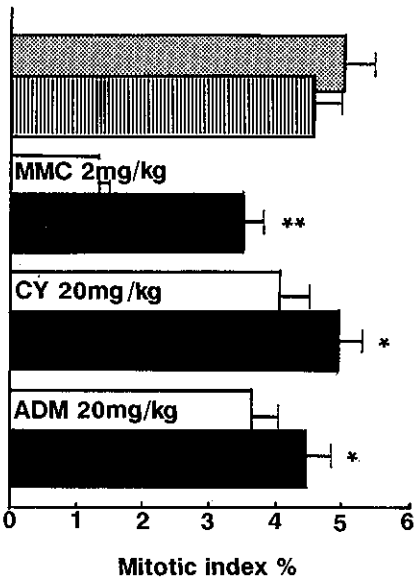

Fig. 3. Effect of sizofiran on suppression of bone marrow cell mitotic index by anticancer agents. Four animals were used in each treatment group and one thousand cells from each animal were scored for mitotic indices. Anticancer agents and sizofiran (10 mg/kg) were administered by the i.v. and i.m. routes, respectively, at the indicated doses. ▨ PBS control; ▥ sizofiran (SPG; 10 mg/kg) alone; □ anticancer agent alone; ■ anticancer agent combined with sizofiran. * $P<0.05$ as compared with anticancer agent-treated mice calculated by using Student's t test.

cated that irradiation with 3 Gy alone markedly suppressed the mitotic indices concomitantly with enhancement of the SCE frequencies from day 1 after treatment. The mitotic indices tended to recover to normal levels by day 7 after irradiation with 3 Gy. The return of SCEs to normal levels after irradiation was delayed and SCEs remained high in frequency until at least day 7 after irradiation (Fig. 4). The results in mice treated with irradiation plus sizofiran are shown in Fig. 5 and Fig. 6. Sizofiran markedly restored the mitotic indices suppressed by irradiation and prevented an increase in the number of SCEs. The inhibitory effects of sizofiran depended on the timing of administration. The present results showed that the maximum protective effects can be expected when sizofiran is administered immediately or soon after irradiation.

DISCUSSION

SCE analysis is often used to detect genetic damage induced by chemotherapy or radiotherapy in bacteria and mammalian cells *in vitro* and *in vivo*.[1-4] In the present study, we examined the inhibitory effects of sizofiran on SCEs induced in bone marrow cells by anticancer agents or irradiation. In addition, the mitotic index was investigated. The frequencies of SCE in mice treated with MMC, ADM or CY were significantly increased compared to PBS-treated controls one day after the treatments, and the mitotic index was observed to decrease. The SCE frequencies were markedly reduced when chemotherapy was combined with sizofiran. The mitotic indices were significantly enhanced in these groups.

We also analyzed the SCEs after irradiation. The highest rate of SCEs was observed on day 3 to 5 post-irradiation with a gradual decrease thereafter. In the present study to investigate the inhibitory effects of sizofiran on the increased frequency of SCEs in irradiated mice, bone marrow cells were taken on day 3 post-irradiation from mice treated with differentially timed sizofiran. The results demonstrated that sizofiran significantly inhibited the increased levels of SCEs and markedly restored the suppressed bone marrow cell mitosis induced by radiation when sizofiran was administered immediately or soon after irradiation.

It has been widely reported that CY is an alkylating agent which produces highly reactive carbonium ions that react with electron-rich areas of susceptible mole-

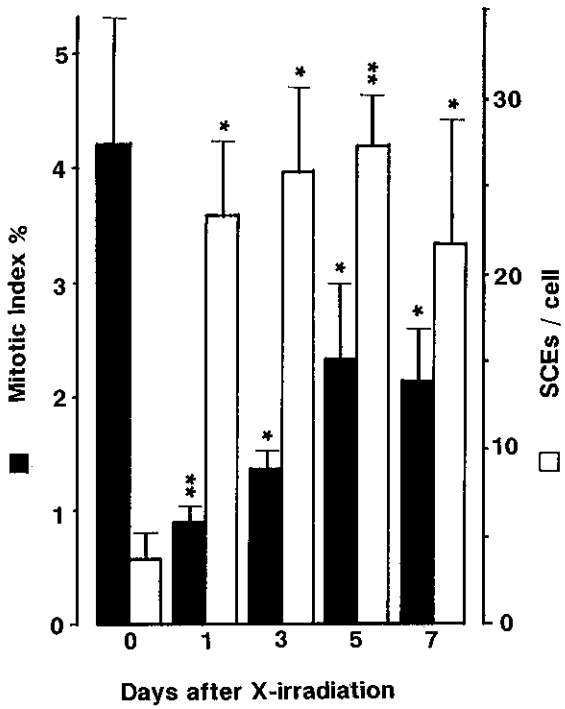

Fig. 4. Chronological changes of SCEs and bone marrow cell mitotic index after irradiation. Four animals were used in each treatment group and 25 second-division metaphases from each animal were scored for SCEs. Mitotic indices were scored in one thousand cells from each mouse. Mice were exposed to whole-body irradiation at the dose of 3 Gy. * $P<0.05$, ** $P<0.001$ as compared with the untreated mice calculated by using Student's t test.

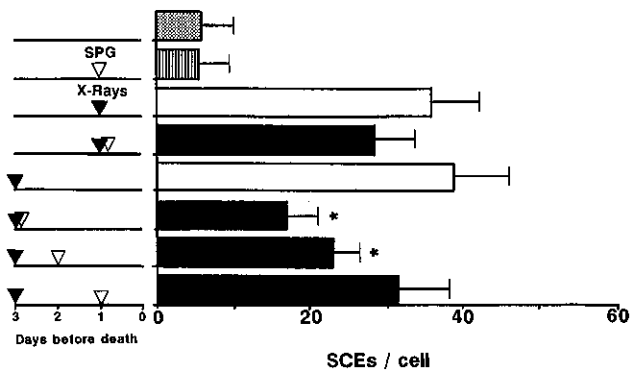

Fig. 5. Effect of sizofiran on irradiation-induced SCEs in mouse bone marrow cells. Four animals were used in each treatment group and 25 second-division metaphases from each animal were scored for SCEs. Sizofiran (SPG 10 mg/kg) was i.m. administered. Mice were exposed to whole-body irradiation at the dose of 3 Gy. * $P<0.05$ as compared with irradiated mice calculated by using Student's t test.

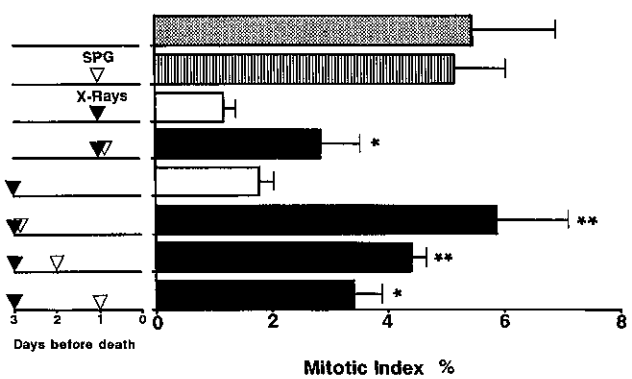

Fig. 6. Effect of sizofiran on suppression of bone marrow cell mitotic index by irradiation. Four animals were used in each treatment group and the mitotic indices were scored in one thousand cells from each animal. Sizofiran (SPG 10 mg/kg) was i.m. administered. Mice were exposed to whole-body irradiation at the dose of 3 Gy. * $P<0.05$, ** $P<0.001$ as compared with the irradiated mice and calculated by using Student's t test.

cules, such as nucleic acids and proteins. MMC is a natural antibiotic which exhibits cellular cytotoxicity by directly interacting with the cellular DNA through the formation of an active species which binds covalently to DNA, producing a variety of genetic alterations such as base alkylation and cross-linking. On the other hand, it is known that ADM or X-rays increase superoxide levels in cells, producing extensive DNA strand breaks. Therefore, it is generally agreed that chromosome breaks are induced by these anticancer agents and irradiation and as a result, cells enter mitosis without fully completing DNA replication.

Thus, the mechanisms of the anticancer action of these agents and irradiation are different from each other and the mechanisms of SCE induction by these agents or irradiation are still unclear, although all the treatments have the potential to lead to DNA damage. So it is difficult to establish how sizofiran inhibits the SCEs induced by the treatments. As we previously reported, sizofiran significantly enhances the ^{3}H-thymidine incorporation of bone marrow cells of 5-fluorouracil-treated mice,[19] and colony formation of the cells in the presence of interleukin-3 and interleukin-6 *in vitro* is augmented by sizofiran administration.[21] Namely, sizofiran accelerates the recovery of various types of antitumor effector cells damaged by chemotherapeutic agents. The present results reveal that sizofiran enhanced mitotic indices depressed by anticancer agents or irradiation. The inhibitory effects of sizofiran on SCE levels in this study may be relevant to the marked restoration of bone marrow

cells. But, regardless of the time of death after irradiation, the most significant recovery was observed when sizofiran was administered simultaneously with irradiation (Figs. 5 and 6). This observation indicates that sizofiran may more actively affect the induction process of SCE. As aggravation in patients given sizofiran combined with chemotherapy or radiotherapy is not observed clinically or experimentally,[22,23] and sizofiran does not affect the growth of various tumor cell lines *in vitro*,[24] it is considered that sizofiran does not itself accelerate the mitotic indices of tumor cells.

Recently, sizofiran has been introduced clinically as an immunopotentiator in patients with cervical cancer after radiation[22] and its host-mediated anticancer activities have been reported.[12,14-19] Our present findings may suggest one more mechanism of efficacy of sizofiran in tumor-bearers treated with chemotherapy or radiotherapy, i.e., the prevention of increase in chromosomal damage induced by treatment with anticancer agents and/or irradiation. Sizofiran may be useful for not only anticancer therapy when combined with anticancer agents or irradiation but also alleviation of bone marrow toxicity. Therefore, we also consider that sizofiran may slow the development of heterogeneity and progression of tumor cells and the development of secondary malignancies caused by chemotherapy or radiotherapy. This possibility should be examined in further studies.

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