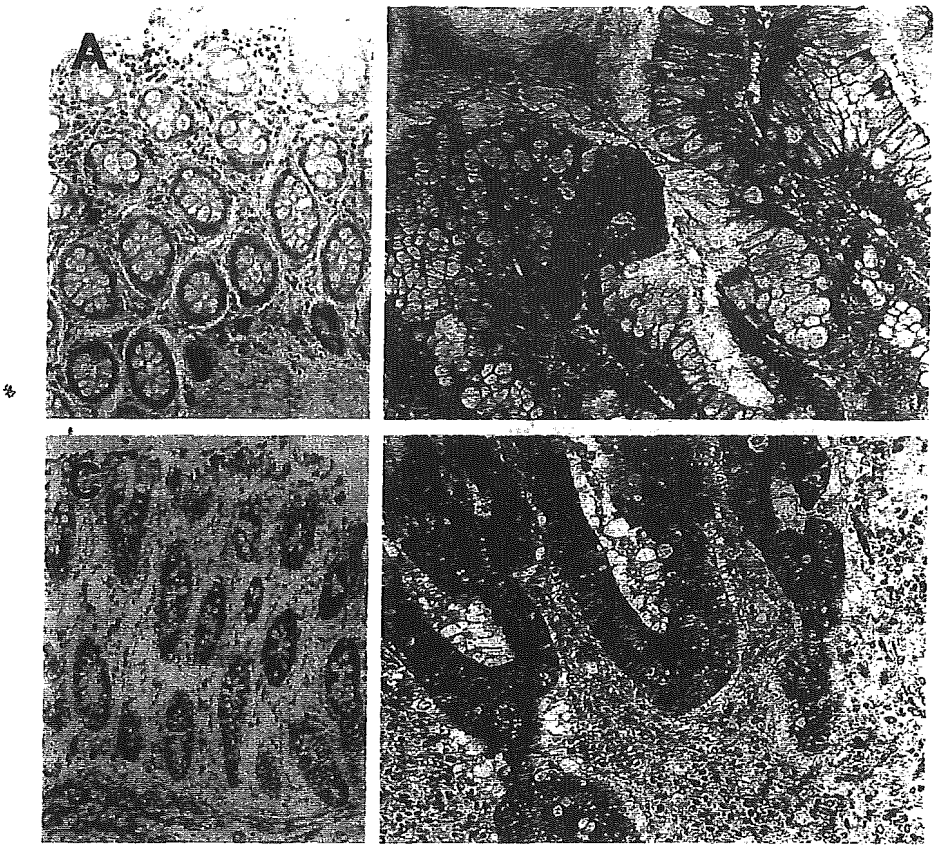

Fig. 2A–D Immunohistochemical localization of survivin in the colon from normal mucosa (**A**) (×100), well-differentiated adenocarcinoma of a young patient (**B**) (×200), and normal mucosa (**C**) (×100), well-differentiated adenocarcinoma of a elderly patient (**D**) (×200). The expression of survivin was observed in a few scattered cells of the normal colonic epithelia, mainly at the bottom of the glands (**A, C**). The subcellular distribution was dominantly cytoplasmic. In contrast, survivin-positive cells were diffusely distributed in cancerous tissues, and staining was cytoplasmic at the cellular level (**B, D**)

Immunohistochemical localization of survivin in colon cancers

To investigate the distribution of survivin, immunohistochemical staining was performed using normal colonic mucosa and cancerous tissues. As shown in Fig. 2A (young) and C (elderly), survivin was mainly detected in the bottom of the glands of normal mucosa, and subcellular localization was mainly cytoplasmic. The staining intensity of positive cells in normal mucosa varied between different samples. In contrast, the majority of samples from colon cancer showed a diffuse localization of survivin, although the staining intensity varied from case to case. At the cellular level, survivin signals in colon cancer cells were predominantly localized in the cytoplasm. The distribution of survivin was similar in well-differentiated and moderately differentiated adenocarcinomas as well as in cancers from young and elderly patients (Fig. 2B, D). Tissue sections that reacted with preimmune rabbit antibody with irrelevant specificity showed no significant staining for any of samples (not shown).

Discussion

For the expression of survivin in colon cancer, a previous study revealed its overexpression in cancer tissue while normal colonic epithelia exhibited weak signals [7]. However, little is known about the potential roles of IAPs in the homeostasis of normal epithelia as well as the pathogenesis of colon cancers among different age groups. In the colon cancer samples in the present study, cancerous tissues had a tendency to exhibit higher levels of survivin but lower levels of cIAP1, cIAP2, and NAIP than the normal mucosa. Immunohistochemical staining revealed a high degree of survivin expression in many cancer cells in the majority of cases, although in normal mucosa, the number of positive cells was small. Thus, differences in the positive cell ratio influenced the intensity and overall expression of survivin mRNA. Kawasaki et al. [7] reported that the expression of survivin correlated with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. We also confirmed the progression-related overexpression of survivin by our findings that moderately differentiated adenocarcinomas expressed higher levels of survivin than well-differentiated adenocarcinomas. Well and moderately differentiated adenocarcinomas, cancerous tissues from elderly patients, demonstrated a significantly higher degree of survivin expression than those from young patients. It would be possible that chances for genetic mutations might increase associated with progression of cancer as well as aging and might result in dysregulation in controlling survivin expression. However, further studies are necessary to determine whether age-related overexpression and differentiation-related

overexpression of survivin in colon cancers occurs through the same mechanisms.

In addition to its antiapoptotic function, survivin also plays a role in the regulation of cell cycle progression during mitosis [8]. The highly proliferative activity and low frequency of apoptosis in colon cancer cells is associated with the significant expression of survivin [7]. We recently reported that the proliferative activity and apoptotic frequency of cancer cells from colorectal cancers exhibited a positive correlation with age [17]. However, in the present study, survivin expression in colon cancers was significantly higher in elderly patients than in young patients. Thus, increased apoptosis in cancers of elderly patients is not attributable to a lack of antiapoptotic regulatory mechanisms by survivin. The manner of age-related changes in the expression of survivin might be associated with the proliferative activities of colon cancers rather than apoptosis. We can also postulate the other possibility that increased apoptosis in cancers of the elderly might be controlled by the caspase-independent mechanisms and, thus, survivin would be overexpressed as the feedback mechanisms of the cells.

Wild-type p53, and not mutant p53, represses survivin expression at the mRNA and protein level [18]. Modification of chromatin within the survivin promoter would explain the silencing of survivin gene transcription by p53 [19]. On the other hand, the overexpression of exogenous survivin protein rescues cells from p53-induced apoptosis in a dose-dependent manner, suggesting that the loss of survivin in part mediates the p53-dependent apoptotic pathway [19]. As there is a high frequency of p53 mutations in many solid cancers, p53 mutation may play a role in controlling the overexpression of survivin in colon cancer. Regarding the age-dependent changes in expression of tumor tissues, a microarray experiment revealed an activation of p53 and some of the genes controlled by p53 at more advanced age [20]. Thus, higher expression of survivin might be induced in response to the increased p53-dependent apoptosis in cancers of the elderly group. However, mutations/loss of p53 gene may occur more frequently with aging in certain settings [21] and, therefore, the p53-associated mechanisms controlling aging and carcinogenesis are complicated.

Using an in vitro cell culture system of colon carcinoma cell lines, Wang et al. [22] reported that the expression of cIAP2 was induced by PKC/NF-κB-dependent pathways. However, the present study revealed that cIAP2 expression was not elevated in colon cancers in vivo. Instead, cIAP1, cIAP2, and NAIP exhibited lower expression in cancerous tissues than in normal mucosa. Further studies are necessary to clarify the function of these molecules in the colonic mucosa as well as in carcinogenesis of the colon.

Signaling pathways involved in survival responses may attenuate the apoptosis response to the cytotoxic tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in colon carcinoma cell lines. Some lines are sensitive, while others are resistant to TRAIL-induced apoptosis [23, 24]. The mechanisms of this resistance include blocking caspase processing by XIAP. However, in the present study, changes in XIAP expression were not notable among normal/cancerous tissues, well/moderately differentiated adenocarcinomas, and cancers of young/elderly patients. Thus, XIAP function in in vivo colon cancer might not be essential for the survival of cancer cells.

In conclusion, we demonstrated the differentiation-related and age-related overexpression of survivin in colon cancer samples. Further studies are warranted to clarify the regulatory mechanisms of IAP expression in colon cancer in association with the apoptotic/proliferative signaling pathways.

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Cytological basis for enhancement of radiation-induced mortality by Friend leukaemia virus infection

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Abstract.
Purpose: To analyse the cytological basis for enhancement of radiation-induced mortality by Friend leukaemia virus infection.
Materials and methods: Cellularity in haematopoietic tissues of C3H mice infected with FLV and/or whole-body irradiation was examined.
Results: When mice were treated with a sublethal dose (3 Gy) of irradiation at 1 week after virus infection, most manifested a severe loss of cellularity in the spleen, bone marrow and peripheral blood 2 weeks after irradiation. More than 90% of the mice died within 1 month post-irradiation. However, this deleterious effect of virus infection on the survival of irradiated mice was observed only when they were irradiated at around 1 week after virus inoculation. Strain differences in the sensitivity to this effect were observed among virus-sensitive strains of mice.
Conclusions: The results indicate that Friend leukaemia virus infection can cause enhancement of radiation sensitivity of haematopoietic cells in host animals in a restricted manner in terms of genetic background and the interval between infection and irradiation.

1. Introduction

Exposure of animals to ionizing radiation causes physiological changes that, depending on the exposure dose, lead to death by haematopoietic and/or intestinal damage. Although it is generally accepted that radiation sensitivity is primarily determined by the intrinsic property of radiosensitivity and the initial number of stem cells (Korn and Kallman 1956, Yuhas and Storer 1962, Van Bekkum 1991, Mori *et al.* 1994), it can also be influenced by epigenetic factors. For example, it is known that microbial infection is an important factor in death associated with radiation, and that it can be controlled to some extent by treatment with antibiotics (Miller *et al.* 1950, Bennet *et al.* 1951). The critical role of bacterial infection in intestinal and haematological radiation deaths has been confirmed by the fact that the germ-free state provides increased radioresistance measured by survival (McLaughlin *et al.* 1964).

It has been recognized that living organisms are exposed to numerous natural and man-made agents that interact with molecules, cells and tissues, and that the combined exposures to radiation and other environmental factors must be taken into account when conducting risk assessments (UNSCEAR 1982, 2000). Viruses are one of the common environmental factors for humans. However, the combined effect of virus infection with radiation has been less well understood. Recently, the relationship of virus infection with the induction of apoptosis has become a topic of interest, since human immunodeficiency virus (HIV) infection is believed to kill cells in patients at least partly by an apoptotic mechanism (Corbeil and Richman 1995, Accornero *et al.* 1998). On the other hand, it has been reported that some viruses have incorporated genes that encode anti-apoptotic proteins or modulate the expression of cellular regulators of apoptosis, although the induction of apoptosis of virus-infected cells is an important host cell defence mechanism (Meinl *et al.* 1998).

The Friend leukaemia virus (FLV) is a murine retrovirus that can cause splenomegaly and induce erythroleukaemia in susceptible mice. FLV infection usually causes anti-apoptotic features in transformed cell lines (Kelly *et al.* 1998, Pereira *et al.* 1999, Quang *et al.* 1999). On the other hand, Shen *et al.* (1988) reported a curative effect of split low-dosage total-body irradiation on FLV-induced leukaemogenesis in DBA mice, suggesting that a decrease in the number of immunosuppressive $CD8^+$ T-cells may be related to the curative effect of irradiation. However, Kitagawa *et al.* (2002) observed a contradictory effect of FLV infection on survival of another FLV-susceptible strain, C3H mice, namely that FLV infection strongly enhances radiation-induced mortality of C3H mice. Therefore, virus infection might affect radiation in different ways.

The present study further characterized the effect

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of virus infection as a modification factor on radiation-induced mortality. The results indicated that the enhancing effect of FLV infection observed was manifested in a restricted manner in terms of genetic background and the interval between infection and irradiation.

2. Materials and methods

2.1. *Mice*

C3H/HeMsNrs (C3H, H-2^k, Fv-2^s), B10/Sn (B10, H-2^b, Fv-2^r) and BALB/c-Fv-4^r (C4W, H-2^d, Fv-4^r) male mice, produced in the present authors' own colonies at the Animal Production Facility of their Institute, were used at 10–15 weeks of age throughout the study. DBA/2Cr Slc (DBA/2, H-2^d, Fv-2^s) and CBA/N Slc (CBA/N, H-2^k, Fv-2^s) mice were purchased from Shizuoka Laboratory Animal Cooperation (Shizuoka, Japan). All mice were maintained in a microbiologically clean animal facility. Experiments were performed with the approval of the local committee on animal experiments, established by the National Institute of Radiological Sciences according to the Japanese Law for Handling of Experimental Animals.

2.2. *Virus infection and total-body irradiation (TBI)*

NB-tropic, polycythemic FLV complex, originally a gift from Dr C. Friend, was prepared as described by Kitagawa *et al.* (1986) and inoculated intraperitoneally (i.p.) at a highly leukaemogenic dose of 10^4 PFU/mouse (Kitagawa *et al.* 1999). Mice were then exposed to X-rays with a Pantak X-ray generator (Shimadzu Ltd, Kyoto, Japan) at a dose-rate of 0.7 Gy min^{-1} (irradiation parameters: 200 kVp, 20 mA, 0.5 mm Cu/0.5 mm Al filters) with graded doses (0.5–3.0 Gy) and at various time intervals (3–21 days) after virus inoculation. Mice were whole-body irradiated. Sham-irradiated mice, i.e. not irradiated, were also similarly prepared in each experiment.

2.3. *Measurement of cellularity*

Cellularity of bone marrow and spleen cells was analysed by flow cytometry. Briefly, spleen cells were stained with fluorescein isothiocyanate (FITC)-labelled anti-B220 monoclonal Ab and phycoerythrin (PE)-labelled anti-Thy1.2 monoclonal Ab, and bone marrow cells with FITC-labelled anti-Gr-1 monoclonal Ab and PE-labelled anti-TER119 monoclonal Ab, followed by flow cytometric analysis with FACScan (Becton Dickinson, San Jose, CA, USA) and then data analysis with CELLQuest software. All antibodies were from PharMingen (San Diego, CA, USA). Cell contents in peripheral blood were assessed using the blood cell counter Sysmex K-4500 (Toa Medical Electronics Co. Ltd, Japan).

2.4. *Measurement of haematopoietic stem cells*

The number of multipotent haematopoietic stem cells (spleen colony-forming unit; CFU-S) was examined according to the method of Till and McCulloch (1961). Either 0.5×10^5 or 1.0×10^6 donor bone marrow cells from C3H mice, treated either with FLV inoculation alone or with FLV inoculation followed by irradiation, were injected intravenously into C3H mice that had been irradiated with 8.5 Gy immediately before use. Twelve days later, their spleens were removed, fixed in Bouin's solution and scored for the presence of spleen colonies using an inverted microscope. For each donor, a minimum of five recipients was used.

For the CFU-E (erythroid colony forming unit) assays, bone marrow cells were mixed with Methocult M3334 (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's guide lines. Assays were plated in 35-mm culture dishes in duplicate. CFU-E was scored on day 2.

3. Results

3.1. *Effect of FLV inoculation on the survival of TBI-treated C3H mice*

Kitagawa *et al.* (2002) showed that a sublethal split dose (1.5 Gy × 2) of whole-body irradiation causes death of FLV-infected C3H mice. To examine whether an FLV inoculation had a similar effect on the survival of C3H mice after treatment with a single dose of TBI, C3H mice were irradiated with a sublethal dose of 3 Gy on day 7 after FLV inoculation. Almost all mice died between 2 and 3 weeks post-irradiation (figure 1). These mice exhibited severe atrophy of spleen and bone marrow, and leukopenia and anaemia in peripheral blood, but no diarrhoea. In the unirradiated group, the treatment of FLV inoculation alone induced marked splenomegaly and leukaemia in all of the mice, and they died between days 47 and 91 after sham irradiation (figure 1). When the irradiated mice after FLV infection were injected with normal bone marrow cells immediately after irradiation, they were rescued from acute lethality but finally died of leukaemia with a delayed onset of tumour development of about 100 days post-irradiation (data not shown). C3H mice treated with a

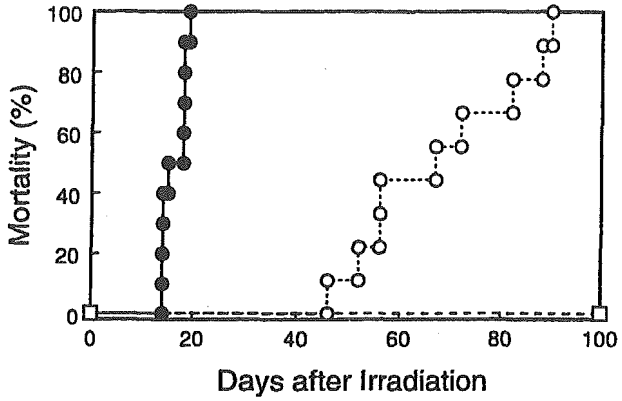

Figure 1. Mortality curve of C3H mice (n = 9) inoculated with FLV alone (open circles) or (n = 10) inoculated with FLV and, seven days later, treated with a sublethal dose (3 Gy) of irradiation (closed circles). Another group of C3H mice (n = 10) inoculated with FLV and irradiated was injected with 10^7 normal syngeneic bone marrow cells just after irradiation (open squares).

sublethal dose (3 Gy) of irradiation alone lived for more than 200 days.

3.2. *Strain difference in the enhancement effect of virus infection on radiation-induced mortality*

Kitagawa *et al.* (2002) showed that C3H mice treated with FLV alone died of leukaemia with splenomegaly from 4 to 8 weeks after irradiation, whereas C3H mice infected with FLV and treated with a sublethal split dose of irradiation (1.5 Gy × 2) died with severe atrophy of the spleen within 3 weeks after irradiation. We also observed that DBA/2 mice treated with FLV alone died of leukaemia with splenomegaly much faster than the C3H mice. In contrast to the case of C3H mice, the treatment with a split-dose of irradiation markedly extended the survival of FLV-infected DBA/2 mice.

To examine the effect of the genetic background of mice on the modifying effect by virus infection for radiation-induced mortality, FLV-sensitive strains of C3H, CBA/N and DBA/2 mice were examined. As shown in figure 2, C3H mice were the most sensitive strain, CBA/N mice were somewhat sensitive and DBA/2 mice were resistant. The mice of these FLV-sensitive strains surviving more than 30 days post-irradiation finally died of leukaemia. On the other hand, there was no mortality following the treatment with FLV inoculation and irradiation in the retro-virus-resistant strains of B10 and C4W mice, in spite of the fact that their resistance was controlled by different genes (B10: Fv-2^r, Fv-4^s; C4W: Fv-2^s, Fv-4^r) (data not shown).

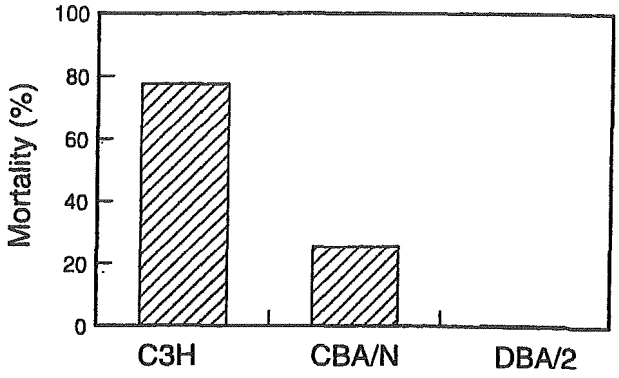

Figure 2. Strain differences in mortality of FLV-susceptible mice FLV-inoculated and treated with 3 Gy of irradiation at 7 days after FLV inoculation. Mortality was examined at 30 days after irradiation (n = 10 or 15 per group). Data represent mean ± SD (vertical lines) of mortality obtained from 4 (C3H), 2 (CBA/N) and 2 (DBA/2) experiments.

3.3. *Effect of radiation dose on mortality of virus-infected mice*

To examine the effect of radiation dose on mortality after a single dose of whole-body irradiation, mice were inoculated with FLV and irradiated at various doses 1 week later. As shown in figure 3, mice died with severe atrophy of spleen at 30 days after irradiation at doses of 1.5 Gy or higher in a dose-dependent manner. The mice surviving 30 days after irradiation died of leukaemia after 40 days post-irradiation.

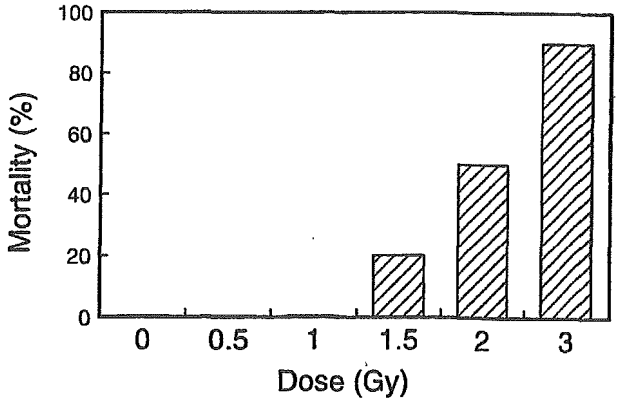

Figure 3. Effect of radiation dose on mortality of C3H mice inoculated with FLV and irradiated. Mortality was examined at 30 days post irradiation. Mice (n = 10 per group) were inoculated with FLV and, seven days later, treated with different doses (0.5–3.0 Gy) of irradiation.

3.4. *Effect of interval between virus inoculation and irradiation*

Various time intervals between FLV inoculation and irradiation (3 Gy) were used to determine the relationship, if any, between interval and enhanced radiation-induced mortality by virus infection. As shown in figure 4(a), only the mice irradiated at 1 week after virus inoculation exhibited high mortality, but the groups irradiated at 3 days or at more than 2 weeks did not. Similar results were obtained in the groups of mice irradiated at 2 Gy (data not shown). The phenomenon was reproducible when the mortality was examined in the groups of mice irradiated at three days, 1 and 2 weeks after virus inoculation. In an experiment with the groups of mice irradiated at days 4, 5, 6, 7, 8, 9 or 10 after virus inoculation (figure 4(b)), it was observed that the groups irradiated at day 5, 6 or 7 had high mortality, those irradiated at day 8 significant mortality, but those at day 4, 9 or 10 no mortality.

To investigate whether the resistance of DBA/2 mice was due to a shift in the time peak of sensitivity, the effect of time interval between FLV inoculation and irradiation on the mortality of DBA/2 mice was also examined. All mice in the groups with various time intervals survived for 30 days after irradiation and finally died of leukaemia (figure 4(c)).

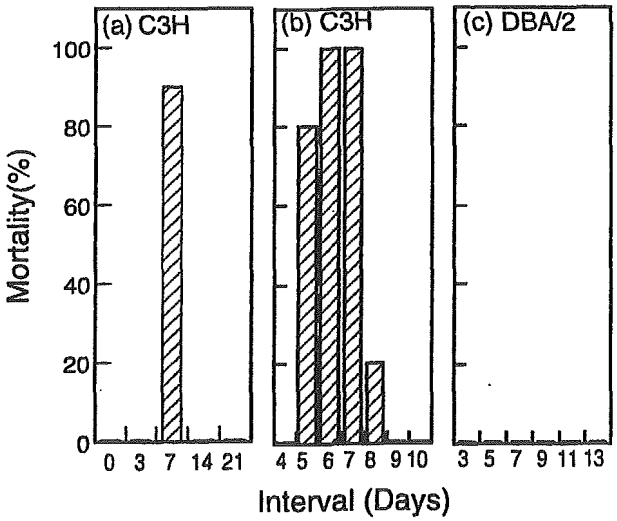

Figure 4. Effect of time interval between FLV inoculation and irradiation on mortality of C3H (a, b) and DBA/2 (c) mice. Mortality was examined at 30 days after 3 Gy of irradiation. Mice (n = 10 per group) were inoculated with FLV and treated with 3 Gy of irradiation at various days after FLV inoculation.

3.5. *Cellularity in peripheral blood, spleen and bone marrow of mice after virus inoculation and irradiation*

The observation of complete rescue with the transfer of normal bone marrow cells strongly suggested that acute mortality following the treatment with FLV inoculation and irradiation was due to haematopoietic failure (figure 1). To investigate further the cause of death in C3H mice that had been infected with FLV and irradiated 1 week later, cellularity in a variety of haematopoietic tissues of the mice was examined at 2 weeks after irradiation when they began to die. C3H mice treated with FLV inoculation and irradiation (FLV + IR group) manifested severe loss of cellularity in white blood cells (WBC) and platelets (PLT) in peripheral blood (figure 5) and in myeloid cells ($Gr\text{-}1^+$) and erythroid cells (TER^+) in bone marrow (figure 6), as compared with those of mice treated with either virus inoculation alone (FLV group) or irradiation alone (IR group). The number of red blood cells in the wasted and dying mice treated with FLV inoculation and irradiation was reduced to less than 20% of that of untreated normal C3H mice. On the other hand, the number of T ($Thy1^+$) and B ($B220^+$) lymphocytes in spleen of the C3H mice treated with FLV inoculation and irradiation was less than that of normal mice but almost the same as that observed in the irradiated-only group (figure 6). Intestinal tissues of the dying C3H mice 2 weeks after irradiation showed normal morphology, just as those of only-irradiated mice (data not shown). In addition, there was no significant abscessing macroscopically in liver and kidney tissues of dead animals. These results suggested that the severe loss of cellularity in peripheral

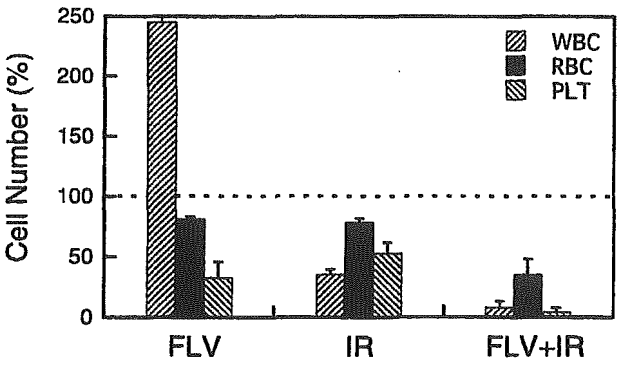

Figure 5. Number of white blood cells (WBC), red blood cells (RBC) and platelets (PLT) in peripheral blood of C3H mice two weeks after irradiation. Mice were treated with either FLV inoculation alone (FLV), irradiation alone (IR, 3 Gy), or FLV inoculation and, seven days later, irradiation (FLV+IR, 3 Gy). Data are shown as the percentage of values observed in untreated normal C3H mice used as control. Sample size was 6 mice in the FLV+IR group and 3 mice each in the other groups. Vertical bars represent SD.

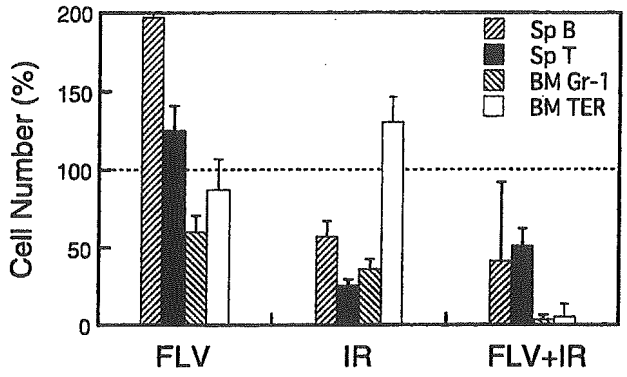

Figure 6. Numbers of Thy 1-positive spleen cells (Sp T), B220-positive spleen cells (Sp B), Gr-1-positive bone marrow cells (BM Gr-1) and TER-positive bone marrow cells (BM TER) of C3H mice two weeks after irradiation. Mice were treated with either FLV-inoculation alone (FLV), irradiation (IR, 3 Gy) alone, or FLV inoculation and, one week later, irradiation (FLV+IR, 3 Gy). Data are shown as the percentage of values observed in untreated normal C3H mice used as control. Sample size was 6 mice in the FLV+IR group and 3 mice each in the other groups. Vertical bars represent SD.

red blood cells might have been the main cause of death in C3H mice.

3.6. *Kinetics of peripheral blood cells in C3H mice irradiated at 1 and 2 weeks after FLV inoculation*

As shown in figure 3(a), only the group of mice irradiated at 1 week after virus inoculation exhibited a high rate of mortality, but the groups irradiated at three days and at more than 2 weeks after FLV inoculation did not. There was no significant change in the spleen weight of C3H mice 3 days after FLV inoculation. However, 2-fold and 4–5-fold increases in spleen weight were observed in the groups at 1 and 2 weeks post-FLV inoculation, respectively, accompanying a preferential increase in the number of TER-positive cells (data not shown). To characterize further the difference in mortality observed in the groups irradiated at 1 and 2 weeks after virus inoculation, we compared the kinetics of peripheral blood cells after irradiation (figure 7). The number of red blood cells (RBC) and white blood cells (WBC) in mice treated with irradiation alone decreased for ten days after irradiation and then began to increase, showing their regeneration. However, in the case of mice irradiated at 1 week after FLV inoculation, they continued to decrease without recovery. On the other hand, the group of mice irradiated at 2 weeks after FLV inoculation showed only about half the decrease in the number of RBC, and there was no significant decrease in WBC.

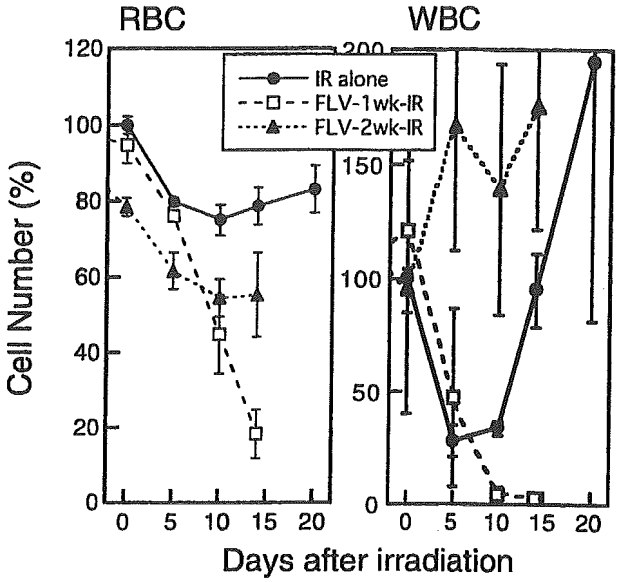

Figure 7. Kinetics of the numbers of peripheral red blood cells (RBC) and white blood cells (WBC) in C3H mice treated with FLV inoculation and irradiation. Mice (n=5 per group) were treated with irradiation (3 Gy) alone at day 0 (IR alone, closed circles), FLV inoculation at day -7 and, one week later, irradiation (FLV-1wk-IR, open squares), or FLV inoculation at day -14 and, two weeks later, irradiation (FLV-2wk-IR, closed triangles). Data are shown as the percentage of values observed in untreated normal C3H mice used as control. Vertical bars represent SD.

3.7. *Number and radiation sensitivity of haematopoietic stem cells in C3H mice infected with FLV*

The observation of leukopenia and anaemia in peripheral blood at 2 weeks post-irradiation in the group of mice irradiated at 1 week after FLV inoculation suggested that haematopoietic progenitor cells are also highly sensitive to the combined treatment of FLV infection and radiation. Therefore, to account for the difference in mortality of the mice irradiated at 1 and 2 weeks after virus inoculation (figure 3(a)), the number and radiosensitivity of CFU-S in bone marrow as a representative haematopoietic stem cell were examined in C3H mice at 1 and 2 weeks after FLV inoculation. The number of CFU-S in bone marrow of both groups of mice treated only with FLV inoculation decreased to less than half of those in untreated mice (-FLV group), but there was no significant difference between these two groups (figure 8(a)). Next, the radiation sensitivity of CFU-S was estimated from the number of CFU-S in bone marrow of C3H mice that were irradiated with 3 Gy at 1 or 2 weeks after FLV inoculation and maintained for one more week for the recovery of stem cells to a detectable level (figure 8(b)). The number of CFU-S

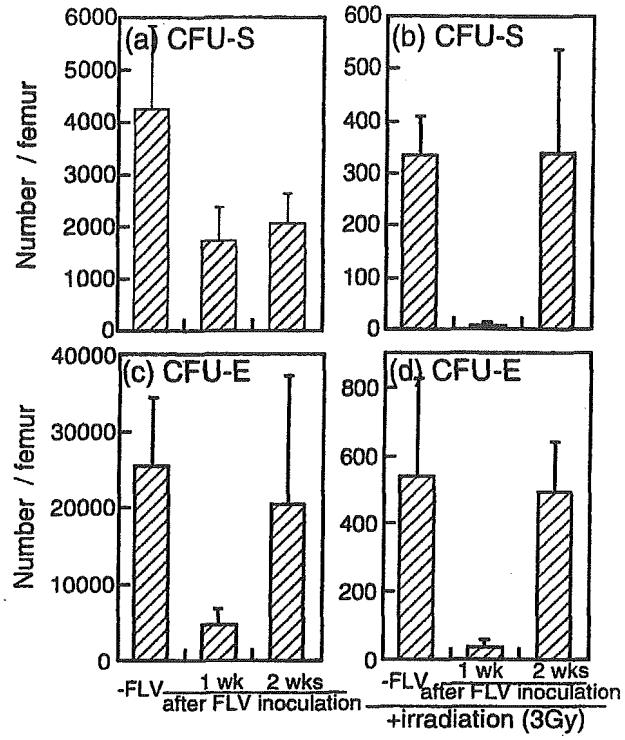

Figure 8. Number and radiation sensitivity of hematopoietic precursor cells (CFU-S and CFU-E) in bone marrow of C3H mice inoculated with FLV. The number of CFU-S was examined in bone marrow cells at one and two weeks after FLV inoculation (a) and the radiation sensitivity of CFU-S was estimated from the number of CFU-S in bone marrow cells of FLV-inoculated mice irradiated with 3 Gy one or two weeks after FLV inoculation and maintained one more week for stem cell recovery (b). Sample size: six bone marrow cells from each of 6 donor mice (a) and four donor bone marrow cells pooled from four or six donor mice in each group (b). Vertical bars represent SD.

The number of CFU-E was examined in bone marrow cells at one and two weeks after FLV inoculation (c) and the radiation sensitivity of CFU-E was estimated from the number of CFU-E in bone marrow cells taken out immediately after 3 Gy-irradiation one or two weeks post FLV inoculation (d). Sample size was six bone marrow cells from each of 6 donor mice (c, d) in each group. Vertical bars represent SD.

was greatly reduced in mice irradiated at 1 week after FLV inoculation, whereas that in mice irradiated at 2 weeks after FLV inoculation was almost the same as that in mice with irradiation only (-FLV group), highlighting the critically higher radiosensitivity of haematopoietic stem cells in mice 1 week post-FLV inoculation.

In addition, the number and radiosensitivity of CFU-E (erythroid colony forming unit) in bone marrow were also examined as precursor cells committed to peripheral RBC, since the severe loss of cellularity in peripheral RBC may be the main

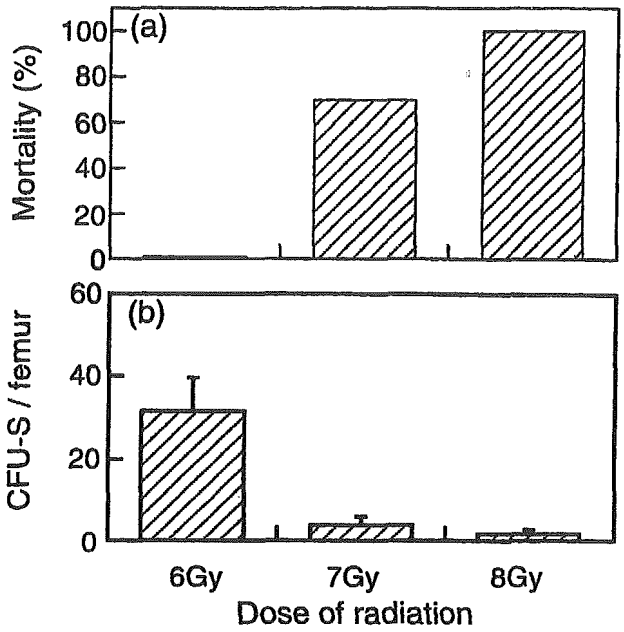

Figure 9. Mortality (a) and number of CFU-S (b) in C3H mice lethally irradiated. (a) Mortality was examined at 30 days post irradiation. Mice (n = 10 per group) were treated with different doses (6, 7, 8 Gy) of irradiation. (b) The number of CFU-S was examined in bone marrow cells at one week after irradiation. Sample size: three bone marrow cells pooled from 4 donor mice in each group. Vertical bars represent SD.

cause of death in C3H mice irradiated at 1 week after FLV inoculation. As shown in figure 8(c, d), the results were similar to those of CFU-S, indicating a higher radiosensitivity of CFU-E in mice 1 week post-FLV inoculation.

3.8. *Mortality and number of CFU-S in irradiated-only mice*

To demonstrate that the reduced level of CFU-S in mice irradiated at 1 week after FLV inoculation could not sustain life (figures 1 and 8), the relationship between mortality and the number of CFU-S was examined with mice treated with various doses of radiation alone. The mice with reduced levels of CFU-S observed in mice irradiated at 1 week after FLV inoculation had acute mortality (groups of 7 and 8 Gy in figure 9), while the mice with a 10-fold higher level of CFU-S (group of 6 Gy) did not show mortality, demonstrating the requirement of such a level of CFU-S to sustain life.

4. Discussion

The purpose of this study was primarily to characterize the cellular events in the enhancement

of radiation-induced haematopoietic injury by FLV infection. When C3H mice were treated with a sublethal dose (3 Gy) of irradiation at around 1 week after virus inoculation, most died around 15 days post-irradiation. Such combined procedures resulted in a severe loss of cellularity in haematopoietic tissues. However, this deleterious effect of virus infection on the survival of mice was only observed when they were irradiated at around 1 week after virus inoculation. Strain differences were also observed in the sensitivity to this effect even among virus-sensitive strains.

With regard to the strain difference in enhancement effect of FLV infection on radiation-induced mortality, C3H mice were the most sensitive strain, CBA/N mice were sensitive to a lesser degree and DBA/2 mice were resistant among the strains sensitive to FLV-induced leukaemogenesis. Since C3H and CBA/N inbred-strain mice were originally developed through a series of crosses between Bagg albinos and the DBA/2 strain (Strong 1942), CBA/N mice should be genetically much closer to C3H than DBA/2 mice. It has been shown that the degree of radiation-induced apoptosis in bone marrow of DBA/2 mice was almost the same as that in C3H mice (Kitagawa *et al.* 2002). However, differences in sensitivity to radiation-induced apoptosis became prominent in the FLV-infected groups of mice.

The present study demonstrated that FLV-infected C3H mice exhibited a marked loss of cellularity especially in erythroid (TER^+) and myeloid cells ($Gr\text{-}1^+$) in the bone marrow 2 weeks after irradiation, whereas uninfected C3H mice had a lesser degree of cellularity loss after irradiation, followed by regeneration. Kitagawa *et al.* (2002) reported that the bone marrow of C3H mice exposed to both FLV inoculation and irradiation showed a significant increase in apoptotic cells, peaking at 12 h post-irradiation, although the treatment with either FLV alone or a sublethal dose of irradiation alone induced minimal apoptosis in bone marrow cells. CFU-S and CFU-E in bone marrow as a representative haematopoietic stem cell and precursor cells committed to erythroid lineage, respectively, also became highly sensitive to radiation 1 week after FLV inoculation. Taken together, these results suggest that the decrease in the number of haematopoietic cells in C3H mice undergoing both FLV inoculation and irradiation was caused by a high level of radiation-induced apoptosis of not only haematopoietic lineage cells themselves, but also their progenitor cells.

In contrast to erythroid (TER^+) and myeloid cells ($Gr\text{-}1^+$) in bone marrow, there was no significant difference in the cellularity of splenic T- and B-lymphocytes 2 weeks after irradiation between C3H mice treated with irradiation alone and those treated with FLV inoculation and irradiation, showing no enhancing effect of virus infection on the radiation sensitivity of lymphocyte populations. On the other hand, as has been known for a long time (Bloom and Bloom 1954, Schrek 1961, Kataoka and Sado 1975), we also observed that splenic T- and B-lymphocytes were highly radiosensitive and their cell number quickly dropped to 30–40% of control in both groups of mice at the first day after the sublethal dose (3 Gy) of irradiation, irrespective of viral infection. Although it is not known why enhancement of radiosensitivity by FLV infection was not induced in lymphocyte populations, it has been shown that receptors for ecotropic murine leukaemia virus are also expressed on splenic T- and B-lymphocytes as well as TER^+ erythroid cells (Suzuki *et al.* 2001).

It is known that FLV infection inhibits various kinds of immune response in infected mice at early stages post-infection (Odaka *et al.* 1966, Mortensen *et al.* 1974). Thus, it is possible that the immunosuppression induced by FLV infection causes a reduced level of production of haematopoiesis-related cytokines like interleukins 2, 3, 4, 5 and 6, which might result in a lesser degree of haematopoiesis and a higher radiation sensitivity of haematopoiesis. However, the relationship between immunosuppression and haematopiesis itself is still controversial. Note that the *scid* (severe combined immunodeficiency) mutation specifically impairs lymphoid but not myeloid differentiation (Dorshkind *et al.* 1984), while interleukin 2-deficient mice develop a haematopoietic disorder characterized by anaemia and neutropenia (Reya *et al.* 1998). Thus, it has yet to be determined whether immunosuppression induced by FLV infection plays a critical role for enhancement of radiation-induced haematopoietic injury of C3H mice by FLV infection.

Mice exhibited severe mortality after the combined treatment with virus inoculation and a sublethal dose of irradiation, but it occurred only when the interval between virus inoculation and irradiation was 5, 6 and 7 days, not when it was shorter or longer (figure 4(b)). The lack of enhancement effect on the mortality of mice treated with irradiation at 3 days after FLV inoculation may be presumed to be due to an insufficient time for the spread of viral infection to a significant population of haematopoietic tissues. With regard to the absence of enhancement of radiosensitivity of haematopoiesis in the group of mice irradiated at 2 weeks after FLV inoculation, it has so far been reported that the total number of CFU-S increased by a factor of 10 in the spleen 2 weeks after virus infection and that the CFU-S probably remain multipotent and manifest radioprotective activity

(Okunewick *et al.* 1972, Okunewick and Phillips 1973). In addition, we observed a higher radiosensitivity of CFU-S and CFU-E in bone marrow of C3H mice 1 week after FLV inoculation as compared with that of CFU-S and CFU-E in bone marrow of normal mice and C3H mice 2 weeks after FLV inoculation (figure 8(b)). The difference in radiosensitivity of CFU-S between the mice 1 and 2 weeks after virus inoculation may significantly influence the regeneration of haematopoietic tissues after irradiation and lead to the fatal outcome of animals treated with a sublethal dose of irradiation after virus inoculation.

However, it is not known why there was a difference in radiation sensitivity of CFU-S and some haematopoietic lineage cells of C3H mice between 1 and 2 weeks after FLV inoculation. FLV infection usually causes anti-apoptotic features in transformed cell lines (Quang *et al.* 1997, Kelly *et al.* 1998, Pereira *et al.* 1999) as well in as primary erythroblasts (Quang *et al.* 1997). In contrast, Kitagawa *et al.* (2002) showed that FLV infection strongly enhances radiation-induced apoptotic cell death of haematopoietic cells, and that the apoptosis occurs through a p53-associated signalling pathway, because p53 knockout mice exhibited a very low frequency of apoptosis in bone marrow after FLV inoculation and irradiation. Daniel *et al.* (1999) reported that DNA-dependent kinase (DNA-PK)-deficient murine scid cells infected with retroviruses showed a substantial reduction in retroviral DNA integration and died by apoptosis, suggesting that the initial events in retroviral integration are detected as DNA damage by the host cells. Thus, the different state of viral genome in the amount of DNA double-stranded ends generated after viral infection might influence the radiation sensitivity of the host cells. However, it is clear that further study of the molecular mechanism responsible for the enhancement of radiation-induced apoptosis and mortality by FLV infection will be required to clarify the strain difference and time dependence of the enhancement of radiation-induced haematopoietic injury by FLV infection. It should also be noted that the evaluation of the molecular mechanism for the enhancement of radiation-induced apoptosis by virus infection is important for clarifying the significance of virus infection for radiation risk in a human population.

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Leptosins isolated from marine fungus *Leptoshaeria* species inhibit DNA topoisomerases I and/or II and induce apoptosis by inactivation of Akt/protein kinase B

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DNA topoisomerases (topo) I and II are molecular targets of several potent anticancer agents. Thus, inhibitors of these enzymes are potential candidates or model compounds for anticancer drugs. Leptosins (Leps) F and C, indole derivatives, were isolated from a marine fungus, *Leptoshaeria* sp. as cytotoxic substances. *In vitro* cytotoxic effects of Lep were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based viability assay. Lep F inhibited the activity of topos I and II, whereas Lep C inhibited topo I *in vitro*. Interestingly both of the compounds were found to be catalytic inhibitors of topo I, as evidenced by the lack of stabilization of reaction intermediate cleavable complex (CC), as camptothecin (CPT) does stabilize. Furthermore, Lep C inhibited the CC stabilization induced by CPT *in vitro*. *In vivo* band depletion analysis demonstrated that Lep C likewise appeared not to stabilize CC, and inhibited CC formation by CPT, indicating that Lep C is also a catalytic inhibitor of topo I *in vivo*. Cell cycle analysis of Lep C-treated cells showed that Lep C appeared to inhibit the progress of cells from G_1 to S phase. Lep C induced apoptosis in RPMI8402 cells, as revealed by the accumulation of cells in sub-G_1 phase, activation of caspase-3 and the nucleosomal degradation of chromosomal DNA. Furthermore, Leps F and C inhibited the Akt pathway, as demonstrated by dose-dependent and time-dependent dephosphorylation of Akt (Ser473). Our study shows that Leps are a group of anticancer chemotherapeutic agents with single or dual catalytic inhibitory activities against topos I and II. (*Cancer Sci* 2005; 96: 816–824)

DNA topoisomerases (topos) are essential nuclear enzymes that regulate DNA topology. There are two classes of topos, classes I and II, that differ in their function and mechanism of action.[1–4] Class I enzymes (topo I, EC 5.99.1.2) act by making a transient break in one DNA strand, allowing the DNA to swivel and release torsional strain, changing the linking number by steps of one.[2,4] Class II enzymes (topo II, EC 5.99.1.3) make transient breaks in both strands of one DNA molecule, allowing the passage of another DNA duplex through the gap, changing the linking number by steps of two.[1–3] These enzymes are crucial for cellular genetic processes such as DNA replication, transcription, recombination, and chromosome segregation at mitosis.

It has long been accepted that topos are valuable targets of cancer chemotherapeutics.[2–5] Several classes of topo inhibitors have been introduced into cancer clinics as potent anticancer drugs, including camptothecin (CPT) derivatives (e.g. irinotecan and topotecan) inhibiting topo I[4] and anthracyclines (e.g. doxorubicin and mitoxantorone), epipodophyllotoxins (e.g. etoposide [VP-16], aminoacridines (e.g. m-AMSA) and ellipticines targeting topo II.[4,5] These agents are active in both hematological and solid malignancies. The activity of these agents is thought to result from stabilization of the DNA/topo cleavable complex (CC), an intermediate in the catalytic cycle of the enzymes,[2,5,6] resulting ultimately in apoptosis. A number of new topo inhibitors have recently been reported that do not stabilize CC. Thus, two general mechanistic classes of topo inhibitors, especially for topo II, have recently been described:[7] (1) classical topo 'poisons' that stabilize CC and stimulate single- or double-strand cleavage of DNA, such as CPT and its derivatives, indolocarbazoles for topo I, and TAS-103[8] for topos I and II; and (2) catalytic inhibitors that prevent the catalytic cycle of the enzymes at steps other than cleavage intermediates, such as dioxopiperazines, ICRF-193 and ICRF-154,[7,9,10] aclarubicin,[11] intoplicin,[12] and F11782.[13] Some of these compounds are dual inhibitors of topos I and II. The catalytic inhibitors of topo II, merbarone[14,15] and dioxopiperazines,[9,10] have been extensively studied and have been shown to inhibit the reopening of the closed clamp formed by the enzyme around DNA by inhibiting the ATPase activity of the enzyme, thus sequestering the enzyme within the cell.[7,16,17]

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Abbreviations: CC, cleavable complex; CPT, camptothecin; DEVD-AMC, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl-7-amino-4-methyl-coumarin; DMSO, dimethylsulfoxide; DTT, dithiothreitol; kDNA, kinetoplast DNA; Lep, leptosin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PI3-K, phosphatidylinositol-3-kinase; SDS, sodium dodecylsulfate; topo, DNA topoisomerase; VP-16, etoposide.

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Leptosin (Lep) derivatives, Lep F and Lep C, have been isolated in our laboratory in search of cytotoxic compounds.[18] In this report we describe that both of the compounds exhibited biological activities, such as the inhibition of topos and cytotoxicity against various tumor cells. Leps are strong catalytic inhibitors of topos, Lep C inhibiting topo I and Lep F inhibiting both topos I and II *in vitro*; Lep C targets topo I *in vivo* as well. These compounds have strong growth-inhibiting and apoptosis-inducing activities against human lymphoblastoid RPMI8402 cells and human embryonic kidney cell line 293 cells. Leps F and C inhibit the survival pathway by inactivation (i.e. dephosphorylation) of Akt/protein kinase B (EC 2.7.1.37).

Materials and Methods

Drugs and chemicals

Leps F and C (Fig. 1) were isolated from a marine fungus *Lestoshaeria* sp.[18] CPT was provided by Yakult Honsha (Tokyo, Japan). VP-16 was provided by Bristol-Myers Squibb (Brea, CA, USA). ICRF-193 was obtained from Zenyaku Kogyo (Tokyo, Japan).

Preparation and assay of topos

Recombinant human topo IIα was purified from a baculovirus expression system as described elsewhere.[19] One unit of topo IIα was defined as the minimal amount of activity required to decatenate 0.2 μg of kinetoplast DNA (kDNA). Isolation of murine topo I from Ehrlich ascites tumor cells was carried out essentially as described previously,[20] except that salt extraction of the enzyme from nuclei was with 0.35 M NaCl and the hydroxyapatite column chromatography was skipped. Topo I activity was monitored by relaxation of supercoiled plasmid DNA.[20,21] One unit of topo I was defined as the minimal amount of activity required to relax 0.2 μg of pT2GN plasmid DNA.

Preparation of kDNA

kDNA was isolated from protozoa *Crithidia fasciculata* as described previously[19,22,23] with some modifications.

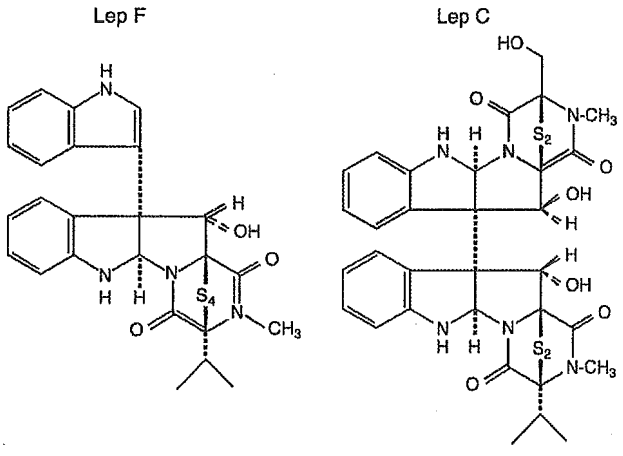

Fig. 1. Chemical structures of Lep F and C.

Inhibitory effect of test compounds on topo II activity was evaluated by detecting the conversion of catenated kDNA to monomer minicircles as described previously.[19,24]

Topo I-mediated DNA cleavage assay

Topo I-mediated cleavable complex formation assay was carried out as described elsewhere[21,25,26] with some modifications. The reaction mixture (20 μL) contained 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5 mM dithiothreitol (DTT), 10% glycerol, 30 μg/mL bovine serum albumin, 10 units of topo I, 0.2 μg of supercoiled pT2GN plasmid DNA and 1 μL of a solution of Leps or CPT as a positive control. Reaction mixtures were incubated for 15 min at 37°C. In experiments of drug combinations, 1 μL each of a test compound and CPT were sequentially added, one before the first incubation at 37°C for 15 min, followed by a further 30-min incubation after the addition of the other, as described in the legend to Figure 3. Then 2.5 μL of 10% sodium dodecylsulfate (SDS) and 2 μL of 20 mg/mL proteinase K were added and the reaction mixtures were digested at 37°C for 1 h. Denatured proteins and drugs were removed by extraction with 1 : 1 mixture of phenol/chloroform. Aqueous phases were taken and mixed with 4 μL of the dye/SDS stop solution and analyzed by electrophoresis on 0.6% agarose gels in the presence of 0.1 μg/mL of ethidium bromide.

Cell culture

Human lymphoblastoid RPMI8402 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. Human embryonic kidney cell line 293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Both of these cell cultures were kept at 37°C in a humidified 5% CO_2 incubator.

Band depletion assay

RPMI8402 cells (2×10^5) in 200 μL of RPMI-1640 medium were prepared in a microcentrifuge tube, treated with each drug or a combination of Lep C and CPT at concentrations described in Figure 4a, and incubated for 15 min at 37°C. For a negative control, we treated cells with 2 μL of dimethylsulfoxide (DMSO) at the same time. Cells were harvested by centrifugation and resuspended in a 10 μL of phosphate-buffered saline (PBS) spiked with two-fold concentrations of the topo inhibitors, as described, to avoid rapid dissolution of the CC. Five μL of 4 × SDS sample buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 2.8 mM 2-mercaptoethanol, 40% glycerol, 0.008% bromophenol blue) was added and immediately sonicated with Sonifier 450 (Branson Ultrasonics, Danbury, CT, USA) to diminish viscosity. Samples were loaded onto 8% SDS polyacrylamide gels, electrophoresed and blotted onto a nitrocellulose membrane. Topo I was detected by antitopo I Scl70 human serum. The membrane was stained with Ponsau S (Sigma, St Louis, MO, USA) according to the manufacturer's instructions.

Drug sensitivity assay of cancer cells

RPMI8402 cells were plated in 96-well plates at an initial density of 2000 cells/well in culture medium. One day after plating, cells were treated with various concentrations of chemotherapeutic agents and cell survival was estimated

by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[27] Three days after the drug treatment plates were centrifuged, the medium was removed and 200 μL of fresh medium containing 250 μg/mL MTT (Sigma) solution was added to each well and incubated at 37°C for 4 h. The plates were centrifuged again, the supernatant was removed and precipitates were dissolved in 150 μL DMSO. The absorbance at 570 nm was measured for each well using a microplate reader (Bio-Rad; Hercules, CA, USA). The IC_{50} values of drugs were calculated from the survival of cells treated for 3 days. IC_{50} was defined as the concentration of drug causing 50% inhibition of cell growth, as compared with the solvent control.

For drug combination analysis, pretreatment of RPMI8402 cells was performed by the addition of Lep C to the cells at various concentrations as indicated. Ten minutes later 20 nM of CPT was added to the culture and incubated for 1 h at 37°C. Then the cells were harvested, washed with PBS and reseeded in 96-well plates at a density of 2000 cells/well. Two days after reseeding, cell survival was estimated by the MTT method.

Cell cycle distribution analysis of cells treated with Lep C

RPMI8402 cells were seeded in 100 mm dishes at an initial density of 5×10^6 cells/dish in culture medium. Twelve hours after plating, the cells were treated with various concentrations of Lep C and other chemotherapeutic agents as indicated. Twenty-four hours after the drug treatment, the cells were fixed with ice-cold 70% ethanol for 30 min, resuspended in 10 μg/mL DNase-free RNase A and 10 μg/mL propidium iodide, and incubated for 30 min at room temperature. After filtration with a nylon cell strainer, cell cycle distribution was monitored with an EPICS Elite (Beckman Coulter, Fullerton, CA, USA).

Caspase assay

Cells were lyzed in caspase lysis buffer (1 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS and 5 mM DTT) and protein concentrations measured by Bio-Rad reagent. Forty micrograms of proteins of cell lysate were incubated with 20 μM acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl-7-amino-4-methylcoumarin (DEVD-AMC; Peptide Institute, Osaka, Japan) in caspase assay buffer (20 mM HEPES, pH 7.4, 10% glycerol and 2 mM DTT) for 60 min at 37°C. AMC released was measured by Fluoroscan Acent FL (Thermo Labsystems, Helsinki, Finland).

DNA ladder formation analysis

Cells (3×10^6) were washed once with PBS, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% Triton X-100) and centrifuged 12 000 *g* for 5 min at 4°C. The supernatant was then treated with 0.2 mg/mL RNase for 1 h at 37°C, followed by treatment with 0.4 mg/mL proteinase K for 30 min at 50°C. The mixture was added with 20 μL of 5 M NaCl and 120 μL of isopropanol and stood overnight at −20°C. After centrifugation at 12 000 *g* at 4°C for 5 min, precipitate was dissolved in 20 μL of TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) added with 4 μL of 6 × loading dye (50% glycerol, 0.1% bromophenol blue), and electrophoresed on 2% agarose gels containing 0.1 μg/mL ethidium bromide.

Western blot analysis

Cells were solubilized with RIPA buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 10% glycerol, 2 mM EDTA).[28] The cell lysates were then subjected to 10% SDS-polyacrylamide gel electrophoresis. The proteins were transblotted onto a nitrocellulose membrane. After blocking, the membranes were incubated with anti-Akt or antiphospho-Akt (Ser473) antibodies (Cell Signaling Technology, Beverly, MA, USA). The membrane was then incubated with an appropriate peroxidase-conjugated secondary antibody and developed with the enhanced chemiluminescence mixture (Amersham Bioscience, Piscataway, NJ, USA).

Results

Lep C inhibits topo I but Lep F inhibits both topo I and II *in vitro*

We tested Leps for inhibition of topo I activity by monitoring the relaxation of supercoiled plasmid DNA, and for inhibition of topo II activity by kDNA decatination assay as described earlier.[19] Both compounds strongly inhibited topo I activity, with IC_{50} values of between 3 and 10 μM for Leps C and F (Fig. 2a). However, inhibition of topo II activity was only observed with Lep F with an IC_{50} value of 10–30 μM. The IC_{50} for Lep C was more than 100 μM (Fig. 2b).

Inhibition of topo I by Leps C and F does not involve significant accumulation of DNA strand breaks

Topo inhibitors are classified according to whether they induce an accumulation of topo-dependent DNA strand breaks as CC or not, reflecting the mechanism of inhibition. We examined whether Leps C and F inhibiting topo I induced

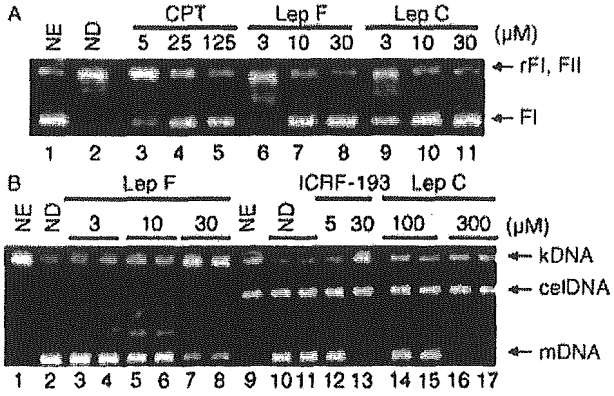

Fig. 2. Inhibition of DNA topoisomerase I (a) and II (b) by Leps F and C. (a) Lane 1, no enzyme (NE) added to the reaction mixture; lane 2, no test compounds (no drugs, ND) were included in the reaction; lane 3, 5 μM CPT; lane 4, 25 μM CPT; lane 5, 125 μM CPT; lane 6, 3 μM Lep F; lane 7, 10 μM Lep F; lane 8, 30 μM Lep F; lane 9, 3 μM Lep C; lane 10, 10 μM Lep C; lane 11, 30 μM Lep C. FI, supercoiled form I; FII, nicked circular form II; rFI, relaxed form I. (b) Lane 1, NE; lane 2, ND; lanes 3 and 4, 3 μM Lep F; lanes 5 and 6, 10 μM Lep F; lanes 7 and 8, 30 μM Lep F; lane 9, NE; lanes 10 and 11, ND; lane 12, 5 μM ICRF-193; lane 13, 30 μM ICRF-193; lanes 14 and 15, 100 μM Lep C; lanes 16 and 17, 300 μM Lep C. celDNA, cellular DNA; kDNA, kinetoplast DNA; mDNA, monomer circular DNA.

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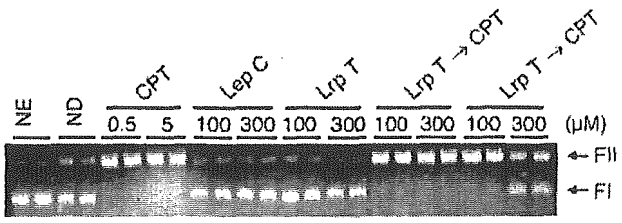

Fig. 3. Inhibition by Lep derivatives of topo I-mediated DNA cleavage induced by CPT. Reaction mixtures containing substrate DNAs and topo I were incubated in the presence of test compounds as described in Figure 2 and under Materials and Methods. Supercoiled FI plasmid DNA was used as the substrate. After the reaction enzyme and test compounds were inactivated, the reaction products were electrophoresed on 0.6% agarose gels.

an accumulation of CC, as described in Materials and Methods. As shown in Figure 3, CPT, a typical topo I poison, induced the accumulation of CC as indicated by an increase in nicked circular form II DNA (FII). No accumulation of FII was observed with Leps C and F up to 300 μM, indicating that Lep is not a poison but a catalytic inhibitor of topo I. It is of great interest to note that Lep C partially inhibited the CC formation by CPT, as illustrated in the two far-right lanes in Figure 3, suggesting that Lep C suppresses the stabilization of CC induced by CPT and that Lep C interacted with topo I in steps other than those of CC formation.

Lep C targets topo I in cultured cells

To quantify DNA–topo CC formed within drug-treated cells, the band depletion assay was employed according to previous reports,[19,29,30] with some modifications. Treatment of human lymphoblastoid RPMI8402 cells with the topo I poison CPT resulted in the depletion of free enzymes detected by Western blotting (Fig. 4a), suggesting the accumulation of topo I-mediated CC within the cells. In contrast, treatment of cells with Lep C, used as a representative Lep with higher potency

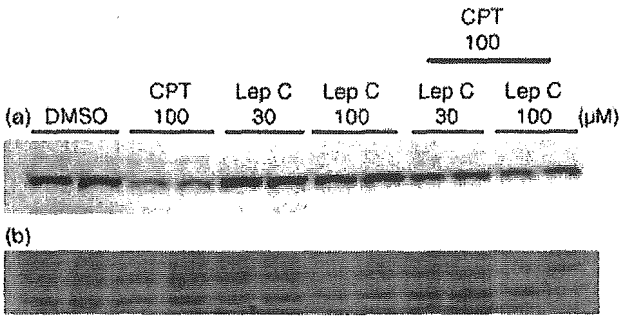

Fig. 4. Band depletion analysis of topo I in RPMI8402 human lymphoblastoma cells. (a) Cells were exposed to drugs singly or in combination, as described under Materials and Methods. Fifteen minutes after incubation, cells were collected by centrifugation and lyzed by addition of SDS-containing lysis buffer, followed by sonication. After protein determination, immunoblotting was performed. (b) It was confirmed by staining the membrane with Ponsau S that the amounts of proteins loaded on the gel were very similar.

of topo I inhibition, did not deplete free enzymes, indicating that Lep C is not a poison like CPT. Treatment of the cells with Lep C prior to CPT tended to restore the free topo I level, as compared with CPT alone, suggesting that Lep C competes with CPT for topo I *in vivo*. This competitive interaction of the two drugs *in vivo* appears to mimic the result obtained in *in vitro* study (Fig. 3). The amount of proteins loaded in each of the lanes of the gel was nearly the same, as shown in Figure 4b.

Lep derivatives inhibit the growth of RPMI8402 cells

The cytotoxicity of Lep C, Lep F, CPT and VP-16 were evaluated with RPMI8402 cells. Dose–response curves were obtained and IC_{50} values were calculated, as shown in Figure 5. The potency of the drugs was in the following order: Lep C > CPT > Lep F > VP-16, as depicted in the figure. RPMI8402 cells exhibited higher sensitivity to Lep derivatives than VP-16 and were nearly as sensitive to Leps as CPT. Examination of the toxicity of Leps F and C on 39 human cancer cell lines established from various tissues from the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan,[31] revealed a similar level of cytotoxicity, with MG-MID (mean logarithm of GI_{50} values, 50% growth-inhibitory concentrations) of −7.41 and −6.8 for Lep C and Lep F, respectively.

Leps F and C induce apoptosis in RPMI8402 cells

The sensitivity of mammalian cells against CPT cytotoxicity was shown to be the largest in S phase of the cell cycle, or rather it was S-phase-specific,[32–34] so it is of great interest to investigate which cell cycle phase Leps might affect. Analysis of cell cycle distribution of cells treated with Lep C by flow cytometry showed that, compared with the solvent control (Fig. 6a), cells treated with Lep C appeared to be

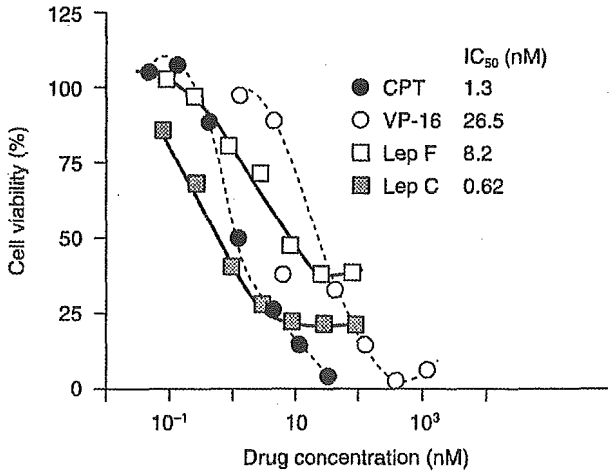

Fig. 5. Cytotoxic activity of Lep derivatives. RPMI8402 human lymphoblastoma cells in 96-well plates were treated with various concentrations of chemotherapeutic agents and cell survival was estimated by the MTT method. The IC_{50} values of drugs were calculated from the survival of cells treated for 3 days. IC_{50} was defined as the concentration of drug causing 50% inhibition of cell growth, as compared with the solvent control DMSO.

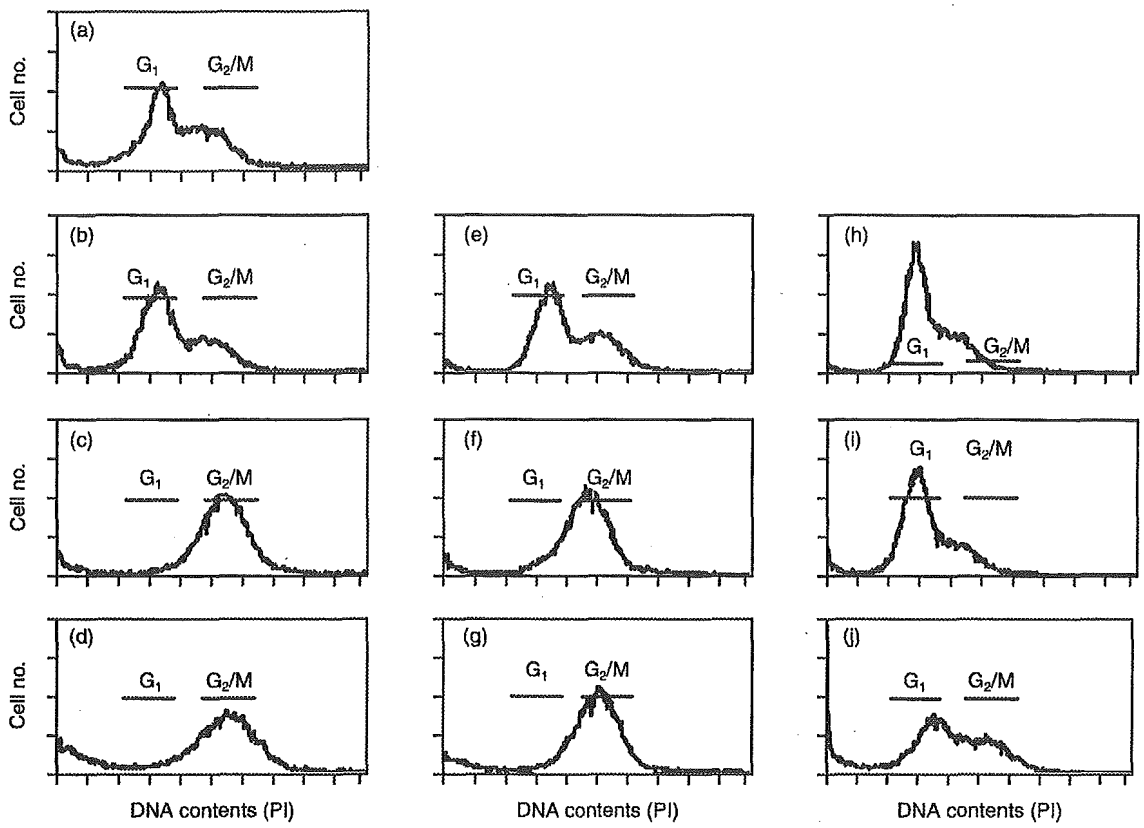

Fig. 6. Cell cycle distribution of cells treated with Lep C. RPMI8402 human lymphoblastoma cells were treated with various concentrations of chemotherapeutic agents for 24 h and flow cytometric analysis was performed, as described in Materials and Methods. (a) solvent control DMSO; (b) 0.02 μM CPT; (c) 0.06 μM CPT; (d) 0.18 μM CPT; (e) 0.3 μM VP-16; (f) 1.0 μM VP-16; (g) 3.0 μM VP-16; (h) 3 nM Lep C; (i) 10 nM Lep C; (j) 30 nM Lep C.

arrested in G_1 phase, as the G_1 cell population increased as the drug concentration was increased up to 10 nM (Fig. 6h,i). However, when the Lep C concentration was increased to 30 nM, the G_1 population decreased and conversely the sub-G_1 fraction increased, indicating that apoptosis took place in high concentrations of the drug. In contrast, cells treated with topo I poison CPT appeared to be arrested in late S to G_2/M phase (Fig. 6b–d). A similar trend of the effect of topo II poison VP-16 arresting cells at late S to G_2/M phase was observed. These experiments clearly indicate that the cellular effects of topo poisons and putative catalytic inhibitors may be different.

We also measured caspase-3 activation as a criterion of apoptosis in Lep-treated RPMI8402 cells using a fluorometric assay, using DEVD-AMC as a substrate. Caspase-3 activity increased after 6 h of treatment with each concentration of drugs (Fig. 7). CPT induced caspase activation maximally at 1 μM. Lep C appears to be stronger than Lep F in caspase activation, as the activity increases dose-dependently with Lep F up to 100 μM, whereas maximum activity was obtained by Lep C at 10 μM. Next, we analyzed DNA strand breaks in Lep-treated RPMI8402 cells. DNA ladder formation indicative of nucleosome-level degradation of DNA, and characteristic of apoptosis, was observed at 6 h after addition of the

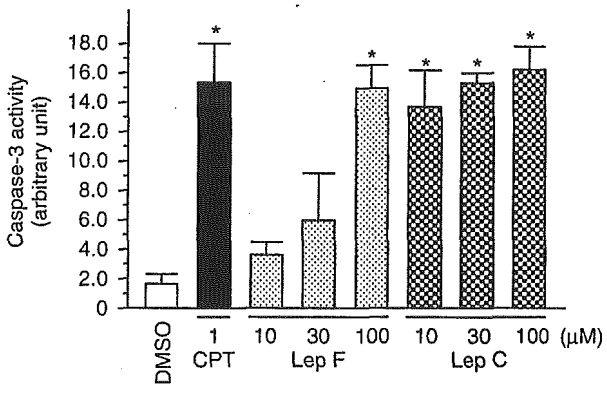

Fig. 7. Activation of caspase-3 by Lep treatment in RPMI8402 human lymphoblastoma cells. Cells were treated with the indicated concentrations of Lep for 6 h. Cell lysates were incubated with the fluorogenic tetrapeptide DEVD-AMC (20 μM) for 1 h at 37°C. The increase in caspase-3 activity in the cell lysates was determined as described in Materials and Methods. The vertical bars represent the standard deviation value of triplicate determinations. Asterisks indicate significant differences from that of the solvent control with *P*-values lower than 0.01.

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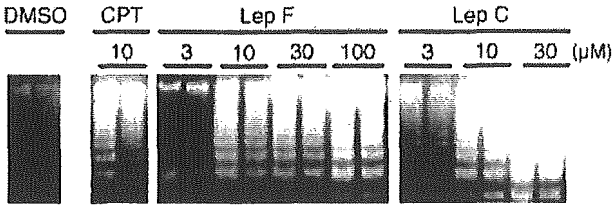

Fig. 8. DNA ladder formation in RPMI8402 human lymphoblastoma cells treated with Lep derivatives. Cells were treated with the indicated doses of the drug for 6 h at 37°C. DNA was extracted and analyzed as described under Materials and Methods.

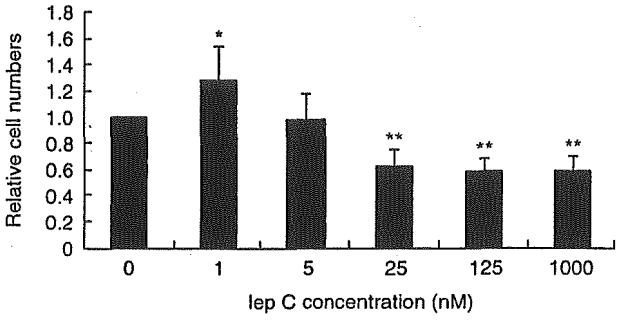

Fig. 9. The effect of combined Lep C and CPT on cell growth. RPMI8402 human lymphoblastoma cells were treated for 1 h with 20 nM of CPT and various concentrations of Lep C. Cell survival was estimated by the MTT method, as described in Materials and Methods. Asterisks indicate significant differences from that of control cells treated with CPT alone. *, $P < 0.01$; **, $P < 0.001$.

drugs (Fig. 8). These results showed that these drugs induced apoptosis, and that the inhibition of cell growth described in Figure 5 was attributed to apoptosis of cells.

Combination of CPT and Lep C treatment showed a synergistic effect on cell growth

To evaluate the effect of a combination of Lep C and CPT on the viability of cells, RPMI8402 cells were incubated with various concentrations of Lep C for 10 min prior to the addition of 20 nM of CPT and 1 h incubation. As shown in Figure 9, in comparison with CPT treatment alone (Lep C 0), in which a 60% reduction in viability was attained, cell growth was inhibited further with increasing concentrations of Lep C up to 1000 nM, although a slight protective effect of a low concentration (1 nM) of Lep C was observed. These results indicated that a combination of CPT and Lep C showed largely an additive or supra-additive effect on growth inhibition.

Lep treatment inactivates Akt by dephosphorylation in 293 cells

Among the known anti-apoptotic pathways, the PI3-K/Akt pathway has been shown to protect cells from pro-apoptotic cues such as radiation and chemicals,[35] and the anti-apoptotic effect of PI3-K is mediated primarily through activation of Akt, one of its downstream targets.[36] Indeed, topotecan, a typical topo I poison, was reported to suppress the Akt signaling pathway.[37] Therefore, we investigated the possibility that Leps induce

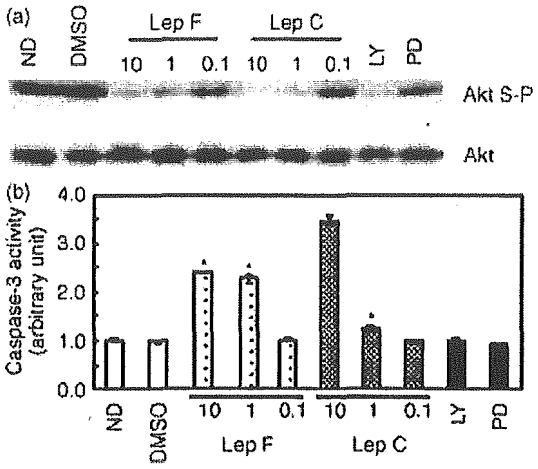

Fig. 10. Dose-dependent inactivation of Akt and activation of caspase-3 in 293 cells treated with Lep derivatives. Cells were treated with the indicated doses of Leps for 24 h. (a) The cell lysates were subjected to Western blot analysis with antiphospho-Akt (Ser473) and anti-Akt antibodies. The concentrations in the figure are in μM. LY, 30 μM LY294002; PD, 45 μM PD98059. (b) Caspase-3 activity in lysates of cells treated as described in (a) was determined as described in Materials and Methods. The vertical bars represent the standard deviation value of triplicate determinations. Asterisks indicate significant differences from that of the solvent control with *P*-values lower than 0.01.

apoptosis through interference with the activation of the protein kinase Akt. As the activation of Akt is mediated by phosphorylation of the kinase on Thr308 and Ser473 residues, we examined the amount of phosphorylated Akt after Lep treatment using an antiphospho-Akt (Ser473) antibody. As shown in Figure 10a, Lep treatment decreased the amount of phospho-Akt in human embryonic kidney 293 cells, as did the PI3-K inhibitor LY 294002. PD 98059, an MEK inhibitor, had no effect on the phosphorylation of Akt. Along with the inactivation of Akt we examined the activation of caspase-3 in the Lep-treated 293 cells using the fluorogenic substrate DEVD-AMC. Lep treatment activated caspase-3 in 293 cells in a dose-dependent manner (Fig. 10b). Time-dependent inactivation of Akt was also observed: concomitant dephosphorylation of Akt and activation of caspase-3 was shown 12 h after treatment (Fig. 11a,b). These results indicate that, in addition to topo inhibition, Lep suppressed Akt kinase activity *in vivo* and stimulated 293 cells to undergo apoptosis.

Discussion

Leps F and C, sulfur-containing indole derivatives, were isolated from marine algal fungus *Leptoshaeria* sp. as cytotoxic substances with antitumor activity.[18] Here we show that these compounds inhibit topos I and/or II: Lep F with an asymmetric structure (Fig. 1) inhibits both topo I and II, but Lep C, with an approximate symmetric structure, inhibits only topo I. The inhibitory activity of Lep C on topo I was higher than that of Lep F (Fig. 2), suggesting that Lep C fits better into the 'left-hand' cleft of the topo I tertiary structure.[38] These results may well be reflected by much higher cytotoxicity of Lep C than Lep F on RPMI8402 cells

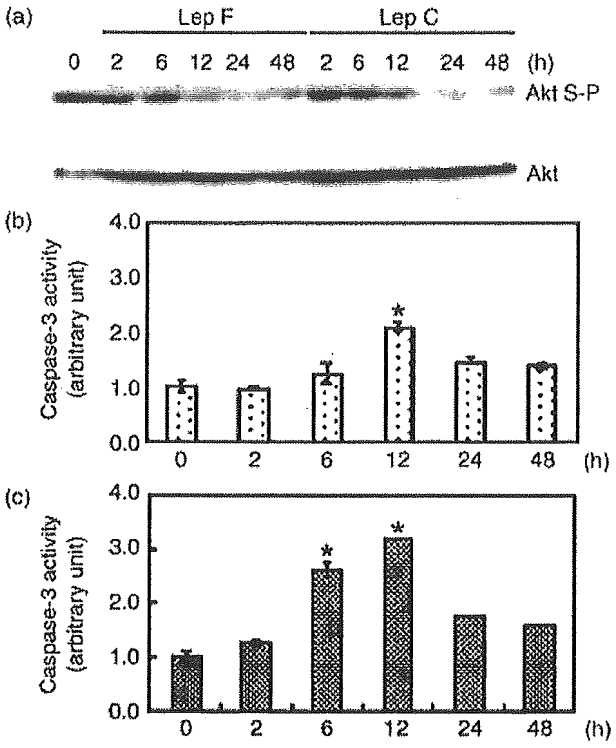

Fig. 11. Time-dependent inactivation Akt and activation of caspase-3 in cells treated with Lep derivatives. Human embryonic kidney cell line 293 cells were treated with 1 μM each of Leps. At the indicated time points, the cells were harvested, some of the lysates were subjected to Western blot analysis and the rest subjected to caspase assay. At time point 0, the cells were harvested before treatment of Leps. (a) Cell lysates were subjected to Western blot analysis with an antiphospho-Akt (Ser473) and anti-Akt antibodies. (b) and (c) Lysates of cells prepared from Lep C (b) and Lep F (c) as in (a) were incubated with the fluorogenic tetrapeptide DEVD-AMC (20 μM) for 1 h at 37°C. Caspase activity was determined as described in Materials and Methods. The vertical bars represent the standard deviation value of triplicate determinations. Asterisks indicate significant differences from that of the solvent control with *P*-values lower than 0.01.

(Fig. 5). Drug sensitivity was similarly assessed by a panel of human cancer cell lines consisting of 39 cell lines established by the Cancer Chemotherapy Center.[31] Both of these derivatives showed strong inhibitory activities, with the MG-MID being −7.41 and −6.8 for Leps C and F, respectively. In parallel with the potency of Lep C on topo I activity, Lep C but not Lep F inhibited CC stabilization induced by CPT (Fig. 3). Furthermore, the result suggests that Lep C interacts with topo I site(s) distinct from that of CPT. Of great interest is the finding that Lep C appeared to interfere with CC stabilization induced *in vivo* by CPT within the nucleus (Fig. 4), implicating that Lep C also targets topo I *in vivo*, as it does *in vitro*. However, the possibility remains that Lep C interferes with CPT-induced CC stabilization by directly interacting with CPT, or, a less likely possibility, Lep C has multiple cytotoxic targets within the cell. It is of great interest to note that COMPARE analysis of the sensitivity fingerprints of Leps F and C with those of standard chemotherapeutics on a panel of human cancer cell lines showed that Leps have little similarity in their mode of action with known topo inhibitors of either class, poison-type or catalytic inhibitor-type agents.

It was clearly shown that inhibition by Leps of growth of cancer cells *in vitro* (Fig. 5) was due to the induction of apoptosis, as revealed by G_1 arrest followed by accumulation of the sub-G_1 phase cells (Fig. 6), activation of caspase-3 (Fig. 7) and DNA degradation (Fig. 8). In regard to cell cycle arrest, both CPT and VP-16 clearly showed G_2/M arrest (Fig. 6), whereas Lep C induced G_1 block. Two mechanisms could explain the difference in the modes of action of these compounds. First, it has been shown that FOXO transcription factors are negatively regulated by the PI3-K-Akt signaling pathway and induce cell cycle arrest in G_1.[39,40] In this study we demonstrated that Lep C led to dephosphorylation of Akt (Figs 10, 11). So it could be considered that Lep C induces G_1 arrest by activating FOXO proteins following the inhibition of Akt phosphorylation. But CPT is also known to suppress Akt phosphorylation,[37] so this mechanism alone is not sufficient to explain the difference in the mode of action of these compounds. The other, more plausible mechanism relates to the difference in the mode of topo I inhibition, that is, CPT being a poison and Lep C being a catalytic inhibitor (Figs 3, 4). Topo I plays an essential role in transcription in eukaryotic cells.[41] CPT has a dual action, DNA damage by stabilizing CC and inhibition of transcription, the former acting dominantly over the other, which in turn mobilizes the G_2 checkpoint, resulting in G_2 arrest. In contrast, Lep C is presumed to have only topo I-inhibitory action without DNA damage and thus transcription inhibition, leading to G_1 arrest. Lep C was stronger in activation of caspase-3 than Lep F, as demonstrated by the near-maximum activation of caspase-3 attained by Lep C at 10 μM, whereas maximum caspase-3 activation by Lep F was attained only at 100 μM (Fig. 7). However, analysis of DNA laddering, which is a marker for apoptotic cell death, demonstrated a similar level of potency in both compounds, that is, induction of laddering by Lep C was observed at 3–10 μM and by Lep F at 10 μM. These results suggest that apoptosis induced by Lep F involves some other signaling pathway(s), such as other executioner caspases or caspase-independent apoptotic pathway(s) in RPMI8402 cells.

There was a considerable discrepancy in IC_{50} values and the effective dosage of Leps between different assays, that is, high dosage in the enzymatic assay and low dosage in the cell growth inhibition assay. Two main reasons could be considered: (1) the growth inhibition shown in Figure 5 might be caused by activating or inactivating multiple signaling pathway(s) following topo inhibition; or (2) in the enzymatic assay, a large amount of substrate DNA is needed to show up the conversion of the substrate, as shown in Figures 2–4, whereas, in the cell-based sensitivity assay, a small fraction of DNA conversion affects the sensitivity of cells, as also shown by other topo-targeting drugs. As shown in Figures 3 and 4, Lep C competed with CPT for topo I in the enzymatic assay. So we investigated whether Lep C suppresses the cytotoxicity of CPT *in vivo*. As shown in Figure 9, and in contrast to expectation, the pre-incubation of cells with Lep C largely enhanced the growth inhibitory effect of CPT. This might also be due to other pathway(s) leading to growth inhibition downstream of the enzymatic lesions.

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Evidence has accumulated showing that various cellular damaging stimuli leading to apoptosis, such as radiation and chemical agents, inactivate the survival pathway, PI-3K/Akt, by the dephosphorylation of Akt.[42–45] Similarly, the CPT derivative topotecan induced apoptosis by the inactivation of PDK-1, the direct upstream activator of Akt, followed by the dephosphorylation and proteolytic cleavage of Akt.[36,46] Thus damage of important cellular components incurred by external stimuli appears to converge on the inactivation of the PI-3K/Akt pathway. In line with these observations, Leps inhibiting topos within the cell were shown to lead to the dephosphorylation of Akt, but the degradation of Akt proteins was not observed by the Lep treatment (Figs 10, 11), as was reported previously.[36,43] In 293 cells, Lep F induced a significant activation of caspase-3 at 1–10 μM (Fig. 10b) in contrast to RPMI8402 cells, where full activation of caspase-3 by Lep F was only observed at 100 μM (Fig. 10), suggesting that apoptotic pathways may differ in different cell types. Furthermore, the interplay between apoptotic pathways (the activation of caspases) and survival pathways (the inactivation of PI-3K/Akt), remains elusive.

Another point of interest is the finding that Lep C induces apoptosis, most likely due to interference with the function of topo I without DNA damage, which strongly suggests that topo I is also essential for the survival and/or growth of mammalian cells in culture, as it is in the development of mouse embryos.[47]

In summary, the results presented here demonstrated that the cytotoxic compounds Leps F and C isolated from a marine fungus, *Leptoshaeria* sp., are a type of topo catalytic inhibitors *in vitro* as well as *in vivo* and that they induce apoptosis in a number of human cancer cell lines. Leps will make a valuable chemical source for anticancer drugs. Furthermore, exploitation of combining and applying this new type of topo inhibitor with conventional poison-type inhibitors has great promise in future cancer chemotherapy.

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