

Therapeutic effects of γ -irradiation in a primary effusion lymphoma mouse model

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Abstract. Primary effusion lymphoma (PEL) is a unique and recently identified non-Hodgkin's lymphoma in immunocompromised individuals. PEL is caused by the Kaposi sarcoma-associated herpes virus/human herpes virus 8 (KSHV/HHV-8) and has a peculiar presentation involving liquid growth in the serous body cavity, chemotherapy resistance and poor prognosis. In search of a new therapeutic modality for PEL, we examined the effect of γ -irradiation on PEL-derived cell lines (BCBL-1, BC-1, and BC-3) *in vitro* and *in vivo*. An MTT assay and trypan blue exclusion assay revealed that irradiation significantly suppressed cell proliferation in the PEL cell lines in a dose-dependent manner, and induced apoptosis. The PEL cell lines were relatively radiosensitive compared with other hematological tumor cell lines (Raji, Jurkat, and K562 cells). Inoculation of the BC-3 cell line into the peritoneal cavity of Rag2/Jak3 double-deficient mice led to massive ascites formation, and subcutaneous injection of BCBL-1 led to solid lymphoma formation. Total body irradiation (4 Gy x 2) with bone marrow transplantation resulted in the complete recovery of both types of PEL-inoculated mice. These results suggest that total body irradiation with bone marrow transplantation can be successfully applied for the treatment of chemotherapy-resistant PEL.

Introduction

Primary effusion lymphoma (PEL) is a unique form of non-Hodgkin's lymphoma that mainly occurs in severely immunocompromised HIV-positive patients (1,2). PEL is etiologically related to human herpes virus-8 (HHV-8) and usually presents as lymphomatous body cavity effusion (pleural, pericardial and peritoneal) in the absence of a solid

tumor mass. Among AIDS-related lymphomas, PEL generally has an extremely aggressive clinical course with a median survival ranging from 2 to 6 months (3). Its prognosis remains poor even after the advent of highly active anti retroviral treatment (HAART) (2). PEL is well known to have strong chemotherapy resistance, and most PEL patients do not respond to combination chemotherapy, such as CHOP. Thus, the optimal treatment for PEL has not yet been defined, and novel effective agents are expected.

Radiotherapy, including total body irradiation, is a crucial treatment option for hematological malignancies, in particular malignant lymphoma (4,5). The treatment of lymphoma depends on numerous factors, including stage, grade, age, and performance status. Since the total irradiation dose for patients is limited, radiotherapy has been used to treat bulky masses, extranodal sites and low-grade stage IA patients.

Here, we examined the radiation sensitivity of PEL cell lines. PEL cells were sensitive to γ -irradiation as compared with other hematological malignant cells. Using an *in vivo* immunodeficient mice model bearing the PEL cell line, we also demonstrated the inhibition of tumor cell growth by total body irradiation. Our findings suggest that radiotherapy can be successfully applied for the treatment of this chemotherapy-resistant malignant lymphoma.

Materials and methods

Cell lines and radiation exposure device. The human PEL cell lines BCBL-1 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) (6), BC-1 (7) and BC-3 (8) (purchased from the American Type Culture Collection, Manassas, VA), and the non-PEL human leukemic cell lines Raji, Jurkat and K562 (obtained from RIKEN Cell Bank, Tsukuba, Japan) were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μ g/ml) in a humidified incubator at 37°C and 5% CO₂. The cells and mice were irradiated at a dose rate of 0.9 Gy/min to a total dose of 1-10 Gy using a ¹³⁷Cs source (Gammacell 40 Exactor; MDS Nordion Inc., Ottawa, Canada).

MTT assay. The antiproliferative effects of γ -irradiation on PEL and non-PEL leukemic cell lines were measured by the

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tetrazolium dye methylthiotetrazole (MTT) method (Sigma, St. Louis, MO). Briefly, 2×10^4 cells were incubated in triplicate in a 96-well microculture plate in the presence of various doses of ionized irradiation in a final volume of 0.1 ml for 48 h at 37°C. Subsequently, MTT (0.5 mg/ml final concentration) was added to each well. After 4 h of additional incubation, 100 μ l of a solution containing 10% SDS plus 0.01 N HCl was added to dissolve the crystal. Absorption values at 595 nm were determined with an automatic ELISA plate reader (Multiskan; Thermo Electron Vantaa, Finland). Values were normalized to untreated (control) samples.

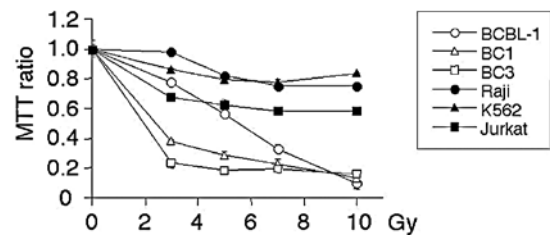
DNA fragmentation assay. To characterize the cell death pattern, DNA ladder assays were performed as previously described (9). Briefly, 1×10^6 cells were lysed in 100 μ l of 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM EDTA and 0.5% Triton X. After centrifugation for 5 min at 15,000 rpm, supernatant samples were treated with RNase A (Sigma) and Proteinase K (Wako Pure Chemical, Osaka, Japan). Subsequently, 20 μ l of 5 M NaCl and 120 μ l isopropanol were added, and the sample was maintained at -20°C for 6 h. Following centrifugation for 15 min at 15,000 rpm, the pellets were dissolved in 20 μ l of TE buffer (10 mM Tris-HCl and 1 mM EDTA) as loading samples. To assess the DNA fragmentation pattern, samples were loaded onto 1.5% agarose gel and electrophoresis was performed.

Flow cytometric analysis of DNA fragmentation. PEL cells were incubated in hypotonic lysing buffer (0.1% sodium citrate, 0.1% Triton X, 0.1% RNase A and 50 μ g/ml propidium iodide (PI) at 4°C for 4 h (10). DNA content in each cell was analyzed on an LSR II flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Annexin V assay. Apoptosis was quantified using the Annexin V-PE Apoptosis Detection kit I (BD Biosciences). Briefly, after γ -irradiation, cells were harvested, washed, and then incubated with Annexin V-PE and 7-AAD for 15 min in the dark, before being analyzed on an LSR II cytometer.

Xenograft and radiotherapy mouse model. Balb/c Rag-2 deficient (Rag-2^{-/-}) mice and Balb/c Jak3-deficient (Jak3^{-/-}) mice were established by crossing Rag-2^{-/-} mice (11) or Jak3^{-/-} mice (12) with the Balb/c strain for 10 generations, respectively. Balb/c Rag-2/Jak3 double deficient (Rag-2^{-/-}Jak3^{-/-}) mice were established by crossing Balb/c Rag-2^{-/-} mice and Balb/c Jak3^{-/-} mice, and were housed and monitored in our animal research facility according to institutional guidelines. Experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Kumamoto University. In a subcutaneous xenograft mouse model, 8- to 10-week-old Rag-2^{-/-}Jak3^{-/-} mice were subcutaneously inoculated with 5×10^6 BCBL-1 cells suspended in 200 μ l PBS. In an intraperitoneal xenograft mouse model, Rag-2^{-/-}Jak3^{-/-} mice were intraperitoneally inoculated with 5×10^6 BC-3 cells suspended in 200 μ l PBS. Seven days after the xenotransplantation of PEL cells, the recipient mice were irradiated (4 Gy x 2), and the bone marrow cells from Rag-2^{-/-}Jak3^{-/-} mice were transplanted into irradiated mice. Tumor burden was evaluated by measuring

A



B

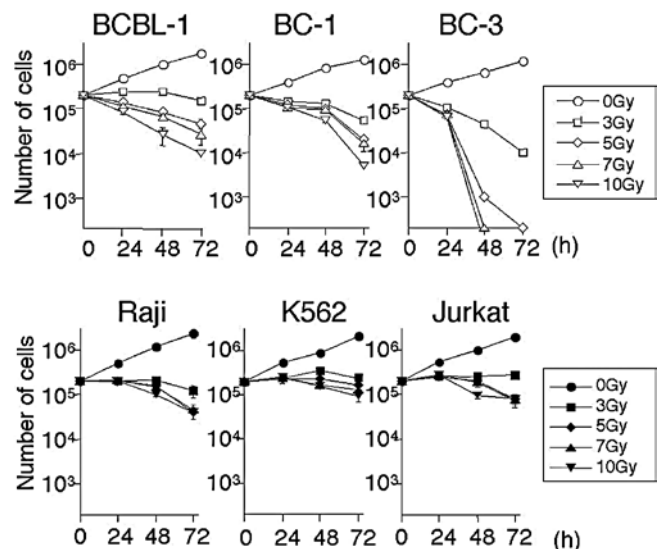


Figure 1. Radiation inhibits the proliferation of PEL cells. (A) PEL cell lines (BCBL-1, BC-1 and BC-3) and non-PEL cell lines (Raji, Jurkat and K562) were irradiated with 3, 5, 7 and 10 Gy and cultured for 3 days. A cell proliferation assay was carried out using the MTT assay (see Materials and methods). One representative result from 3 independent experiments is shown. (B) PEL cell lines (BCBL-1, BC-1 and BC-3) and non-PEL cell lines (Raji, Jurkat and K562) were irradiated with 3, 5, 7 and 10 Gy and cultured for 3 days. Cell counting was carried out every 24 h using a hematology analyzer. A representative result of 3 independent experiments is shown.

the tumor mass body weight, or ascites. Tumor growth was monitored by measuring maximal and minimal diameters with calipers every week, and tumor size was estimated with the formula: tumor size (mm³) = length (mm) x width² (mm) x 0.4, as described previously (13,14). For assessment of disease-free survival, Kaplan-Meier analysis was performed and P-values were determined by 2-tailed analysis with log-rank tests.

Statistical analysis. Assays were performed in triplicate and expressed as the mean \pm SD. The statistical significance of differences observed between experimental groups was determined using the Student's t-test. P-values <0.05 were considered significant.

Results

Inhibitory effect of ionized irradiation on the proliferation of PEL cell lines. Initially, the radiosensitivity of PEL cells was examined. Three PEL cell lines (BCBL-1, BC-1 and BC-3) and three non-PEL cell lines (Raji, B lymphoid; Jurkat,

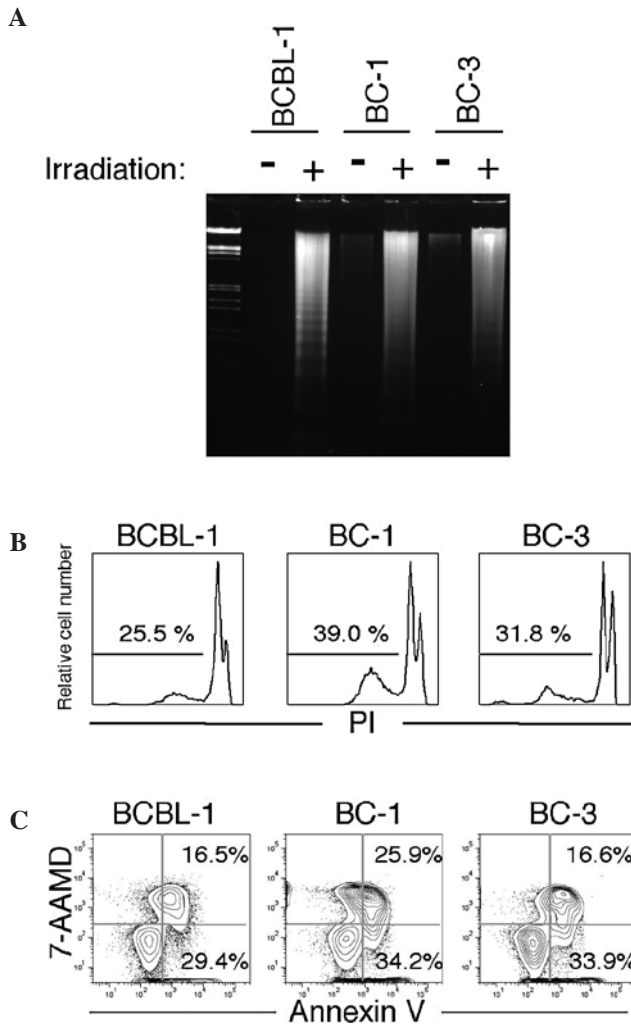


Figure 2. Irradiation induces the apoptosis of PEL cells. (A) Induction of apoptosis in PEL cells by ionized irradiation (ladder formation). Irradiated (5 Gy) or non-irradiated PEL cells were cultured for 48 h. DNA was extracted and separated by electrophoresis on 1.5% agarose gel. (B) Apoptosis was determined by flow cytometric analysis of DNA fragmentation using PI staining. (C) Radiation-induced apoptosis as detected by Annexin V and 7-AAD dual staining. The PEL cell lines BCBL-1, BC-1 and BC-3 were irradiated (5 Gy), cultured for 72 h, and subsequently stained with Annexin-PE and 7-AAD before being analyzed by flow cytometry. A representative result of 3 independent experiments is shown.

T lymphoid; and K562, erythroleukemia) were cultured after irradiation (0, 3, 5, 7 and 10 Gy) for 3 days, and proliferation was analyzed by the MTT assays. As shown in Fig. 1, irradiation inhibited cell growth in a dose-dependent manner in all PEL and non-PEL cell lines. PEL cell lines were more sensitive than non-PEL cell lines to radiotherapy. The effects of irradiation were then confirmed by traditional cell count. PEL and non-PEL cell lines were cultured after irradiation (0, 3, 5, 7 and 10 Gy) for 3 days, and cell numbers were counted by trypan blue staining. As shown in Fig. 1B, the number of PEL cells decreased in a dose- and time-dependent manner. In contrast, non-PEL cells survived, indicating that PEL cells are more sensitive to irradiation.

Induction of apoptosis in PEL cells by γ -irradiation. To determine whether growth inhibition by ionized irradiation

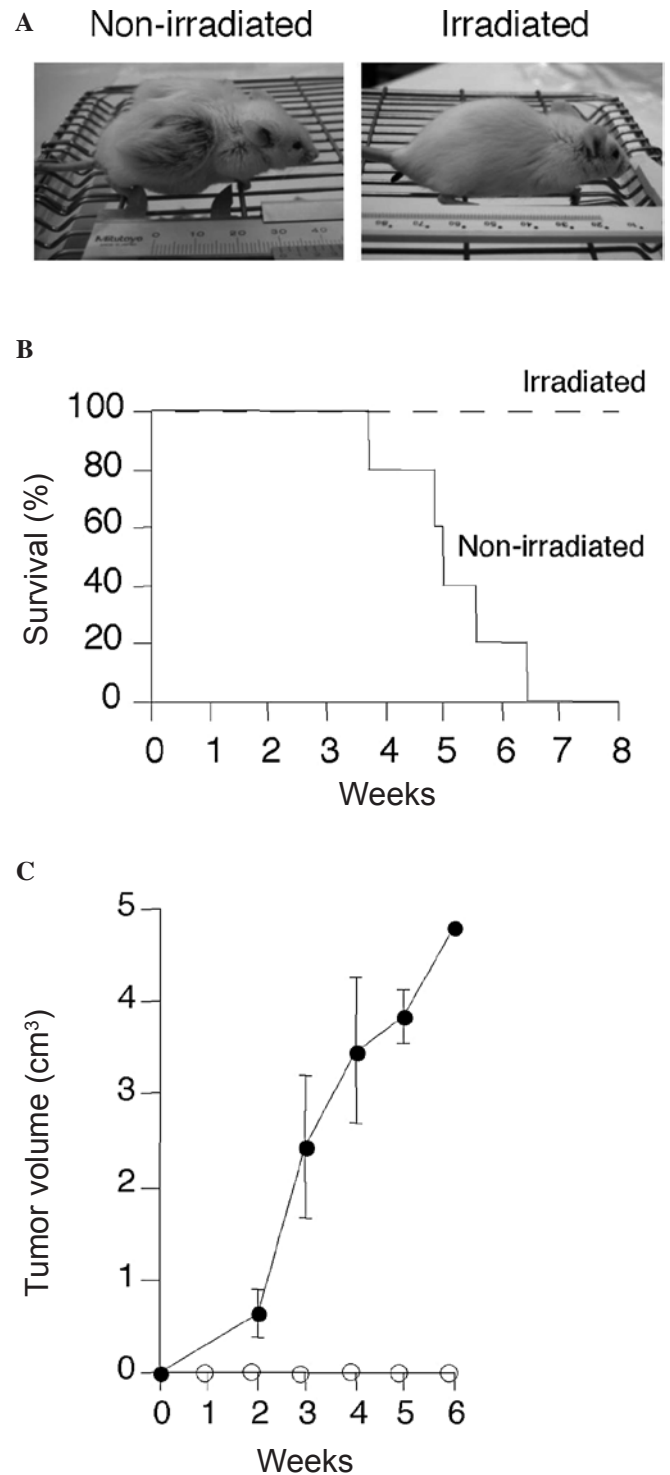


Figure 3. Inhibition of subcutaneously inoculated PEL cell growth by irradiation *in vivo*. (A) Irradiated and non-irradiated mice 4 weeks after subcutaneous inoculation with BCBL-1. (B) Anti-tumor effect of ionized irradiation (survival). All non-irradiated PEL mice died within ~6 weeks, while the irradiated mice survived. (C) Tumor volume of mice inoculated with BCBL-1 cells and irradiated or non-irradiated, expressed as the mean \pm SD from 5 mice and the tumor volume of each mouse ($P < 0.05$).

was attributable to apoptosis in PEL cells, DNA fragmentation analysis (ladder formation and detection of the sub-G1 fraction by flow cytometry) was performed 48 h after ionized irradiation. As shown in Fig. 2A, irradiation caused DNA

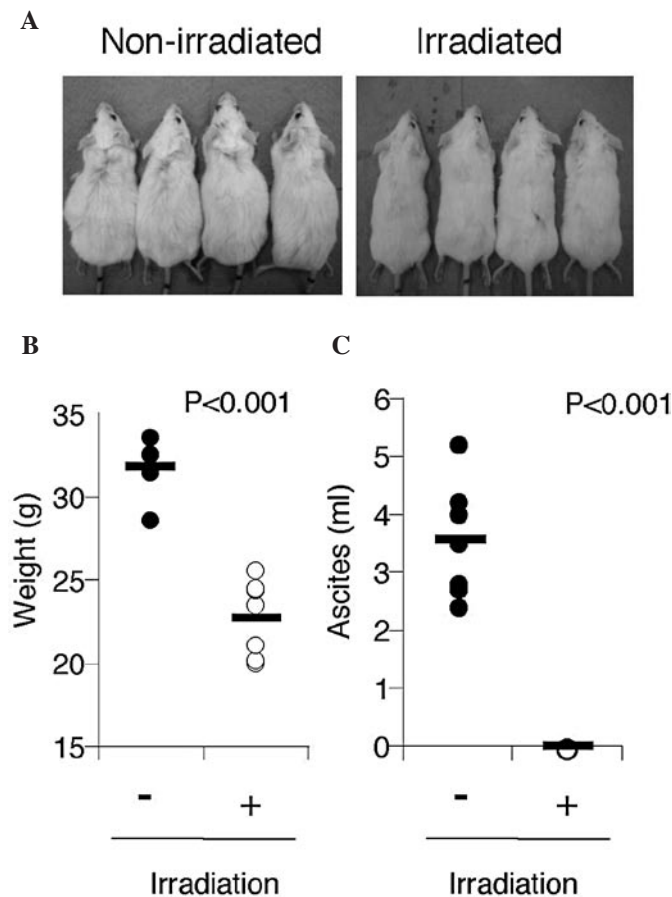


Figure 4. Inhibition of ascites formation in mice inoculated with PEL cells that underwent total body irradiation. (A) Ascites-bearing non-irradiated PEL mice and irradiated PEL mice (6 weeks after inoculation). (B) Body weight of mice inoculated with BC3 cells and irradiated or non-irradiated mice. (C) Volume of ascites inoculated with BC3 cells and irradiated or non-irradiated mice.

fragmentation, which is a characteristic of apoptotic cell death (9). In addition, sub-G1 populations (the apoptotic fraction) were detected in all three PEL cell lines (Fig. 2B).

An Annexin V binding assay was also performed for further confirmation of irradiation-induced apoptosis in PEL cells. The annexin positive 7-AAD negative fraction represents the early phase of apoptosis, whereas the Annexin positive 7-AAD positive fraction represents the late phase of apoptosis and necrosis. (15). As shown in Fig. 2C, irradiation induced apoptosis in PEL cell lines. These results suggest that growth inhibition by irradiation occurs via the induction of apoptosis.

In vivo effects of irradiation on severe immunodeficient mice inoculated with a PEL cell line. Since the above results suggested the efficacy of radiotherapy for the treatment of PEL patients, we next examined the *in vivo* effects of irradiation in an immunodeficient mice model. Severe immunodeficient Rag-2^{-/-}Jak3^{-/-} mice were inoculated subcutaneously with 5x10⁶ BCBL-1 cells. Seven days after the xenotransplantation of the BCBL-1 cells, the recipient mice were irradiated (4 Gy x 2). Total bone marrow cells from Rag-2^{-/-}Jak3^{-/-} mice (1x10⁷/mouse) were transplanted into the irradiated mice. Non-irradiated mice efficiently developed large subcutaneous

tumors (Fig. 3A), exhibited clinical signs of near-death, such as piloerection, weight loss and cachexia, and succumbed within 3-6 weeks of transplantation (Fig. 3B and C). On the other hand, irradiated mice did not develop tumors and survived for more than 3 months after transplantation without developing tumors.

Since the formation of massive effusion is the nature of PEL, Rag-2/Jak3 double-deficient mice were inoculated intraperitoneally with 5x10⁶ BC-3 cells. BC-3 produced massive ascites within 6 weeks of inoculation (3.5±1.0 ml, n=7) (Fig. 4A) and body weight was significantly increased in mice (Fig. 4B), indicating that this was a clinically relevant PEL model.

The irradiated mice appeared to be healthy, with the same body weight as non-tumor inoculated mice, and did not have ascites (Fig. 4B). The body weight of non-irradiated mice was significantly increased by massive ascites compared with that of irradiated mice (31.7±1.6 g vs. 22.8±2.3 g, n=7, P<0.001).

These data indicate that γ -irradiation significantly inhibits the growth and infiltration of PEL cells *in vivo*.

Discussion

In the present study, we investigated the direct effects of γ -irradiation on PEL cells *in vitro* and *in vivo*. PEL cells were relatively radiosensitive compared with other hematological malignant cells *in vitro*, and total body irradiation with bone marrow transplantation rescued PEL-inoculated Rag-2/Jak3 double-deficient mice, preventing the formation of tumors and effusions. Thus, we suggest that γ -irradiation is a promising candidate for chemotherapy-resistant PEL.

PEL is a rare high-grade B-cell malignancy associated with herpes virus HHV-8 infection, and is mainly observed during the course of HIV infection. The outcome of PEL after polychemotherapy, such as CHOP, has generally been poor, even since the advent of HAART. The potential for methotrexate to diffuse in serous cavities suggests the use of high-dose methotrexate in association with a CHOP-derived regimen (16); however, it has been reported that patients with serous effusion might be at increased risk of toxicity following high-dose methotrexate chemotherapy (17). In addition, there are reports in which even intensive treatment regimens were unsuccessful (18). Thus, the optimal treatment for PEL has not yet been defined. HHV-8 contains a homologue of cellular FLIP protein called vFLIP that has the ability to activate the NF- κ B pathway (19), which is known to protect against apoptosis induced by diverse stimuli (19-21). The NF- κ B pathway is known to induce the expression of a number of anti-apoptotic genes, such as Bcl-xL, A1, cIAP1, cIAP2, XIAP and IEX-1 (22). In addition, it has been demonstrated that the inhibition of NF- κ B induces the apoptosis of PEL cells (23,24). vFLIP-mediated NF- κ B activation is necessary for the survival of PEL cells, and may cause them to become chemoresistant. Thus, the NF- κ B pathway represents an appropriate target for the molecular therapy of PEL, and several NF- κ B inhibitors are already potential candidates (24-26). One of these candidates, the proteasome inhibitor bortezomib, has been shown to inhibit NF- κ B activity and induce the apoptosis of PEL cell lines *in vitro* (27,28); however, bortezomib failed to control the progression of PEL in a clinical trial (29), indicating that

preclinical anti-tumor activity does not necessarily translate directly into activity in patients, and that preclinical studies using animal models are required to determine the actual advantage of NF- κ B inhibitors in PEL (29).

Malignant lymphomas are characterized by a high degree of radioresponsiveness. Consequently, radiotherapy is an important modality in controlling these malignancies (30). However, as most lymphomas are systemic diseases that are chemotherapy sensitive, use of radiotherapy has been limited to localized lymphomas. Recently, it was reported that chemotherapy-refractory HIV-associated PEL patients achieved remission and survived for more than 12 months (31). Our findings also suggest that PEL is sensitive to radiation treatment (Fig. 1). In addition, it has been shown that the *in vitro* radiosensitivity of tumor cells correlates with the response to therapeutic irradiation (32). Thus, radiotherapy should be considered as part of the treatment recommendation for patients with chemotherapy-refractory PEL.

In vivo experiments showed that non-treated mice subcutaneously xenografted with PEL developed large tumors, while peritoneally xenografted mice gained body weight and effusion in the peritoneal cavity (Figs. 3 and 4). On the other hand, the irradiated groups did not have either effusions or tumors for 12 weeks, indicating that irradiation is capable of rescuing PEL-xenografted mice. Animal models of human malignancies have been applied to study the nature of cancer stem cells and to assess the therapeutic effects of novel therapeutic strategies against malignant neoplasms (33,34). In particular, the recent introduction of severe immunodeficient mice has enabled us to develop mice mimicking hematologic malignancies (26,35). In this study, we used PEL-xenografted Rag2/Jak3 double-deficient mice resembling the diffuse nature of human PEL, which is quite useful to assess the therapeutic efficacy of γ -irradiation in mice in a hematological malignancy model.

In summary, the present study demonstrated that γ -irradiation is quite effective for the treatment of PEL both *in vitro* and *in vivo*. Our study shows the usefulness of radiotherapy for the treatment of chemotherapy-resistant PEL patients. Radiotherapy should therefore be considered for the treatment of chemotherapy-resistant PEL patients.

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