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Dose and Dose-rate Effects of X rays and Fission Neutrons on Lymphocyte Apoptosis in p53(+/+) and p53(–/–) Mice

KAZUO FUJIKAWA[1]*, YUKINORI HASEGAWA[1], SHINYA MATSUZAWA[1], AKIHIRO FUKUNAGA[2], TETSUO ITOH[1] and SOHEI KONDO[1]

[1]Atomic Energy Research Institute, Kinki University, Higashiosaka 577–8502, Japan
[2]Osaka City University College of Nursing, Osaka 545–0051, Japan
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Following the exposure of mice to X rays or fission neutrons, the frequency (*F*) of apoptosis was measured after 4 h, and the weight loss or lymphocyte content loss in the thymus and spleen was measured after 24 h. In p53(+/+) mice, *F* increased linearly with the dose (*D* (Gy)) and the induced rate per Gy of *F* (detected by TUNEL staining) was 0.05 and 0.23 for X rays and fission neutrons, respectively. Therefore, the RBE of fission neutrons was 4.6 for apoptosis induction. This indicates that radiation-induced apoptosis is mostly due to double strand breaks (DSBs) in DNA because we previously obtained almost the same RBE value of fission neutrons for the induction of crossover mutations in *Drosophila melanogaster*, which arise from the recombinational repair of DSBs. In p53(+/+) mice, decreases in the organ weight and the lymphocyte content were observed for the thymus and the spleen 24 h after X-irradiation. These atrophic changes in the thymus and the spleen quantitatively corresponded to the total apoptotic cell deaths occurring in them. However, in p53(–/–) mice, no vigorous apoptosis was induced after X-irradiation, and hyperplastic changes in the weight and the lymphocyte content appeared in the thymus and the spleen 24 h after X-irradiation. In p53(+/+) mice, there was no difference in the induced rate per Gy of reduction in the surviving fraction of lymphocytes between acute (0.4 Gy/min) and chronic (3 mGy/min) γ-irradiations. Namely, radiation-induced apoptosis in lymphocytes is a dose-rate independent event.

INTRODUCTION

It has been long known (review by Maruyama and Feola[1)]) that after small doses of whole-body X-irradiation of mice or rats, interphase death leading to dead pyknotic nuclei occur rapidly and markedly in lymphocytes of the thymus[2)]; also, the thymus shows rapid shrinkage, and the lymphoid tissues undergo marked degradation[3,4)]. Yamada and Ohyama[5)]

*Corresponding author: Phone; +81–6–6721-2332 (ext. 4426), Fax; +81–6–6721–3743,
E-mail; genken@ned.kindai.ac.jp

presented the first evidence that the interphase death of rat lymphocytes after X-irradiation *in vitro* is due to apoptosis. Nomura et al[6] reported that the number of dying cells in the thymus or spleen by interphase death reached a sharp maximum 4 h after whole-body X-irradiation with low doses from 0.05 to 0.5 Gy and that the dose-response curves were approximately linear. They detected dying cells in specimens of irradiated thymuses or spleens by scoring positive cells after staining with erythrocin B. Erythrocin B stains cells when the specimens were cultured at 37°C, but not at 25°C, indicating that the interphase death was due to the activation of cell-death machinery. Surh and Sprent[7] presented critical evidence that the interphase death of thymic lymphocytes is due to apoptosis; they found a rapid, marked increase in the number of positive cells stained by the TUNEL method in cryostat sections of the thymus from C57BL/6 mice irradiated with 1 Gy. They further reported that the number of apoptotic cells in the irradiated cortex increased within 1 h (but not 30 min) after irradiation, that nearly all apoptotic cells detected after 1–2 h were observed within macrophages, that appreciable numbers of 'free' (not macrophage-associated) apoptotic cells were apparent 4 h after irradiation and that, compared with a normal thymus, the cell yields from the irradiated thymuses were reduced by 68% 24 h after irradiation.

Lowe et al[8] and Clarke et al[9] reported that thymocytes from p53(–/–) mice, when cultured for a short time *in vitro*, were resistant to apoptotic death after γ-irradiation and those from p53(+/+) mice were radiosensitive to apoptosis. Nomoto et al[10] reported that upon whole-body X-irradiation with 3 Gy, apoptotic cells detected by the TUNEL method increased rapidly to a maximal frequency of 80% in the neural tube of fetal p53(+/+) mice 4 h after irradiation, and p53(–/–) fetal mice showed no increase in apoptotic cells. Neither the p53 mRNA nor the p53 protein level increased after irradiation. Thus, they proposed that radiation-induced apoptosis in a mouse fetus is not a transcriptional event. However, in cultured human embryonic cells, the p53 protein level increases after X-irradiation[11]. Norimura et al[12] reported that upon X-irradiation of mouse fetuses at a midgestational stage, p53(+/+) mice showed a 50% incidence of anomalies and a 60% incidence of fetal deaths, whereas p53(–/–) fetuses showed a higher incidence (70%) of anomalies, but a much lower (7%) incidence of fetal deaths. Based on these findings, they proposed that fetal tissues have a p53-dependent guardian, which is capable of an apoptotic elimination of cells with unrepaired DNA damage from the irradiated tissues. It is, however, difficult to directly test this hypothesis because the teratogenic process leading from the initial event of radiation-induced DNA damage on gestational day 9.5 to the endpoints 9 days later is complicated. Therefore, in the present study, we established a short-term test for an endpoint of radiation-induced apoptosis.

Firstly, we compared the difference in the sensitivity to the radiation-induced apoptosis of p53(+/+) and p53(–/–) adult mice. We used two different methods to detect apoptotic cells: haematoxylin-eosin (HE) staining and TUNEL staining. Secondly, we attempted to answer two questions: (1) what is the primary cause of radiation-induced apoptosis and (2) is there a short term endpoint that represents cumulative effects of all cell deaths via apoptosis that takes place promptly after irradiation in the thymus or spleen? The short-term endpoint for apoptosis used here was atrophic changes in the thymus and spleen 24 h after irradiation. With regard to the first question, we measured the relative biological effectiveness (RBE) of fission

neutrons for the induction of apoptosis, because if the primary cause of radiation-induced apoptosis is double strand breaks (DSBs) in DNA, the RBE of fission neutrons for apoptosis induction is expected to be about 5. This is because we previously obtained an RBE of 5.1 for fission neutrons for the induction of crossover mutations in *D. melanogaster*, which arise from the recombinational repair of DSBs[13]. Hendry et al[14] reported that the RBE of fast neutrons (14–600 MeV) for the induction of apoptosis in the small intestinal crypt stem cells *in vivo* was 3 to 4. Meijer et al[15] reported 1 to 3 for the RBE of accelerated nitrogen ions (32–45 MeV) for the induction of apoptosis in human lymphocytes *in vitro*.

MATERIALS AND METHODS

Mouse strains and irradiation with X rays, γ rays or fission neutron

Female mice of ICR and C57BL/6 strains were obtained from Simizu Laboratory Supplies (Kyoto, Japan), whereas p53 null female mice, i.e., a p53(–/–) derivative of the C57BL/6 strain with the commercial name TAG-p53^{R}, were imported from Taconic (NY, USA). Unless otherwise stated, mice were used at 7–9 weeks of age at the time of irradiation and were housed 4 or 5 per cage in polycarbonate solid-bottom suspended drawer-type cages containing Clean-Chip hardwood bedding (Clea Japan Inc., Tokyo) and were provided with diet FM (Oriental Yeast Co., Tokyo) and tap water ad libitum. The light cycle was 12 h light and 12 h dark.

Mice were subjected to whole-body irradiation with various doses of X rays, γ rays or fission neutron-γ mixed radiation available inside a nuclear reactor installed at Kinki University. The reactor used is called the University Teaching and Research Reactor, type B, and was manufactured in 1958 by Advanced Technology Laboratory (a division of American-Standard), Mountain View, California (see Ayaki et al[13] for details).

The dose-rates given were 0.3 Gy/min for X-irradiation, 0.4 Gy/min or 3.3 mGy/min for γ-irradiation and 3 tissue-mGy/min for fission neutron irradiation.

For X-irradiation, a Hitachi X-ray generator was used at 140 kV and 4 mA with a 1.0 mm Al filter. The dose rate was measured with a Victoreen chamber. For γ-irradiation at dose-rates of 0.4 Gy/min or 3.3 mGy/min, we used the ^{60}Co or ^{137}Cs irradiators installed at Hiroshima University. The dose-rate was measured using the tertiary standardized ionization chamber in Japan. For irradiation with fission neutrons from the reactor at Kinki University, mice housed inside polycarbonate tubes were placed at the center of the reactor's core, and the reactor was operated at 1 watt. The dose-rates of fission neutrons and γ rays at the irradiated place in the reactor were 0.20 tissue-Gy/h, and 0.20 Gy/h, respectively. These dose-rate values were obtained using a pair of a fast neutron-sensitive ionization chamber, IC-17P, made of a polyethylene-walled cylindrical chamber filled with ethylene gas, and a fast neutron-insensitive, though γ-ray sensitive, ionization chamber, IC-17G, made of a graphite-walled cylindrical chamber filled with CO_2 gas (Far West Tech. Inc., CA, USA)[13,16].

Measurement of the frequency of apoptotic cells in thymus and spleen in vivo

Four mice were irradiated in each experiment with different doses of radiation. The mice were sacrificed 4 h later by anesthetization with dimethyl ether, and their thymuses and spleens were then removed. Each thymus or spleen was cut into two halves; one half was fixed in Bouin's solution and embedded in paraffin wax. Transverse 4-μm sections were cut and stained with haematoxylin and eosin (HE). The other half of the organ was fixed in 10% neutral formalin, paraffin-embedded and 4-μm sections were stained using the ApopTag DNA nick end-labeling kit (Oncor, Inc., USA), which is a modified TUNEL method[17]. Sections were counterstained with haematoxylin and eosin. Stained sections were microscopically inspected at a magnification of $\times$1000 for apoptotic lymphocytes in the cortex region of thymus (Fig. 1) and the white pulps of spleen. The frequency of apoptotic cells was calculated as the number ratio of apoptotic cells detected to total lymphocytes inspected.

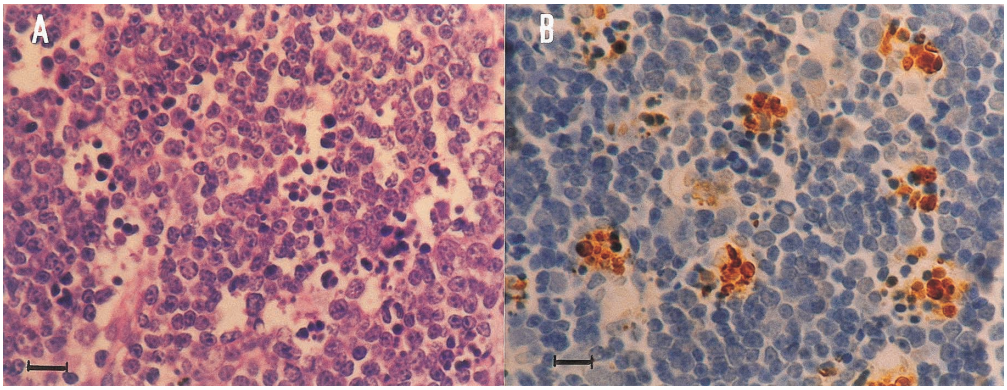

Fig. 1. Specimens of the irradiated thymus 4 h after X-irradiation with 2 Gy, (**A**) fixed in Bouin's solution and stained with haematoxylin-eosin (HE) to make visible the cells with pyknotic nuclei (see the dark foci), and (**B**) stained by the TUNEL method to make visible the cells containing fragmented DNA (see clusters of brownish-yellow cells engulfed by macrophages). The 10 μm bars are indicated.

Measurement of atrophic changes in thymus and spleen by a reduction in the organ weight or lymphocyte content

Four to five mice exposed to various doses of radiation were sacrificed 24 h after irradiation. The thymuses and spleens were then removed, individually weighed and the lymphocyte content of each organ was measured by the following method. Each organ was held in a 60 mm $\times$ 15 mm polystyrene Petri dish containing a 10 ml aliquot of a physiological saline solution (Wako Pure Chemical, Osaka) between two layers of stainless-steel mesh and gently pressed with the tip of a gloved forefinger from the upper side of the mesh sheets in order to release lymphocytes from the organ into the solution until only the organ capsule remained inside the mesh sheets. Released cells were filtered through a sheet of nylon gauze and then stained with Tuerk's solution (Katayama Chemical Ind, Osaka, Japan). The number of nucleated cells stained 'violet' in the Tuerk's solution was counted microscopically using a Buerker-Tuerk haematocytometer. The number of 'violet' cells per ml in the suspension is

defined as the relative number density of surviving lymphocytes in each organ.

RESULTS

Upon irradiation, apoptosis and atrophic changes occur in thymus and spleen of p53(+/+) mice but not in p53(–/–) mice

After the whole-body X-irradiation of p53(+/+) and p53(–/–) C57BL/6 mice with 2 Gy, the apoptosis frequency (F), the weight (M) and the lymphocyte content (N) in the thymus and spleen were measured. The results are given in Table 1. As can be seen in the table, not only increases in the apoptosis frequency, but also reductions in the weight and the lymphocyte content of the two organs occurred in p53(+/+) C57BL/6 mice, whereas in p53(–/–) C57BL/6 mice, the apoptosis frequency did not increase at all, but the weight and the lymphocyte content of the two organs increased.

In order to examine whether radiation-induced atrophic changes were exclusively due to radiation-induced apoptosis, we quantitatively compared the induced rate (per unit dose) of the apoptosis frequency (b) with the induced reduction rate (per unit dose) in weight of the thymus or spleen (m) or with the induced reduction rate (per unit dose) in the surviving lymphocytes (k) after the X-irradiation of mice. Namely, the three parameters used are defined as

$$F = a + bD, \tag{1}$$

Table 1. Changes in apoptosis frequency, organ weight and lymphocyte content for the thymus and spleen of p53(+/+) and p53(–/–) C57BL/6 mice after acute X-irradiation

Organ	Genotype of p53 gene	Dose (Gy)	Apoptosis frequency F	Organ weight M (mg)	Lymphocyte content N (10^6 cells)
Thymus	+/+	0	0.0065 (27/4091)	55 ± 8	147 ± 14
	+/+	2	0.36 (1116/3110)[a]	32 ± 4[b]	33 ± 7[b]
	+/+	2	0.0094 (38/4048)[c]	32 ± 3[c]	NT[d]
Thymus	–/–	0	0.0072 (31/4323)	71 ± 20	140 ± 8
	–/–	2	0.0075 (32/4258)[a]	78 ± 4[b]	242 ± 8[b]
	–/–	2	0.018 (74/4098)[c]	69 ± 1[c]	NT[d]
Spleen	+/+	0	0.0093 (40/4285)	67 ± 3	135 ± 18
	+/+	2	0.28 (981/3542)[a]	49 ± 10[b]	52 ± 13[b]
	+/+	2	0.011 (51/4489)[c]	50 ± 1[c]	NT[d]
Spleen	–/–	0	0.012 (52/4349)	83 ± 6	122 ± 27
	–/–	2	0.041 (185/4545)[a]	88 ± 20[b]	162 ± 21[b]
	–/–	2	0.017 (77/4483)[c]	94 ± 15[c]	NT[d]

[a] Measured by HE staining 4 h after X-irradiation

[b] Measured 24 h after X-irradiation

[c] Measured 72 h after X-irradiation

[d] NT = not tested

where F is frequency of the cells dying by apoptosis measured 4 h after irradiation by HE or TUNEL staining, a the spontaneous frequency of apoptotic cells, b the rate per Gy of induced apoptotic cell frequency by radiation and D (Gy) the radiation dose.

$$(M - M_r) = (M_o - M_r) \exp(-mD), \tag{2}$$

where M is the weight of the thymus or spleen 24 h after irradiation, M_o the M value before irradiation, M_r the weight of the apoptosis-resistant fraction of the organ, and m the rate per Gy of the radiation-induced reduction in the weight of the thymus or spleen.

$$N / N_o = S = \exp(-kD), \tag{3}$$

where N is the total number of lymphocytes in the thymus or spleen 24 h after irradiation, N_o the N value before irradiation, S the survival and k the rate per Gy of radiation-induced reduction in survival S of the lymphocytes.

The values of b, m and k calculated from the observed F, M and S values using Eqs. 1 to 3 are given in Table 2. As can be seen in the line 'Rrs' of Table 2, the b value is about one third to one fourth of the m or k value. This means that about three- or four-times the fraction of cells dying by apoptosis 4 h after X-irradiation with 1 Gy corresponds quantitatively to the fraction of the weight loss or the lymphocyte content loss from the thymus or spleen 24 h after X-irradiation with 1 Gy.

Table 2. Induced apoptosis rate per Gy (b), induced reduction rate per Gy in organ weight (m) and induced reduction rate per Gy in the lymphocyte content (k) for the thymus and spleen of C57BL/6 mice after whole-body X-irradiation (see text for definition of b, m, k, F, M, M_r, N, N_o)

Organ	Dose (Gy)	F	b (Gy^{-1})	$M - M_r$[a] (mg)	Ratio[b]	m (Gy^{-1})	N (10^6 cells)	Ratio[c]	k (Gy^{-1})
Thymus	0	0.007		(55 – 22) ± 8	1		147 ± 14	1	
	1	NT[d]		(41 – 22) ± 2	0.56	0.58	61 ± 9	0.41	0.89
	2	0.36	0.18	(32 – 22) ± 4	0.30	0.62	33 ± 7	0.22	0.76
	Average		0.18			0.60			0.83
Rrs[e]	$b : m : k$		1			3.3			4.6
Spleen	0	0.009		(67 – 27) ± 3	1		135 ± 18	1	
	1	NT[d]		(53 – 27) ± 2	0.46	0.46	73 ± 8	0.54	0.62
	2	0.28	0.14	(49 – 27) ± 10	0.55	0.60	52 ± 13	0.39	0.47
	Average		0.14			0.53			0.55
Rrs[e]	$b : m : k$		1			3.7			3.9

[a] The M_r value was estimated from the relation of M versus the dose given in Fig 4.

[b] Ratio = $(M - M_r)/(M_o - M_r)$

[c] Ratio = N / N_o

[d] NT = not tested

[e] Rrs = Relative ratios

Relative biological effectiveness of fission neutrons for the induction of apoptosis in the thymus

Studies of the relative biological effectiveness (RBE) of fission neutrons versus X or γ rays were carried out using female ICR mice. Mice were whole-body exposed to X-ray doses of 0.5, 1, 2, 4, 7 and 10 Gy at 0.3 Gy/min and sacrificed 4 h later. The thymuses were removed and used for scoring apoptotic cells by HE or TUNEL staining. After HE staining, apoptotic cells were defined as those that contained pyknotic nuclei; their frequency was slightly higher than that detected by TUNEL staining for the existence of fragmented DNA (Figs. 1 and 2).

To calculate the RBE values, we used the linear portions of the empirical F versus D curves given in Fig. 2. The corresponding data points for apoptosis detected by TUNEL staining are redrawn in Fig. 3, where the highest D value is limited to 2 Gy. As can be seen in Fig. 3, the F versus D relation is linear. Therefore, this portion of the F versus D curve can be accurately described by Eq. 1. Similarly, the apoptosis frequency (F) increased linearly with the dose of neutron-γ mixed radiation from 0.2 to 0.8 Gy (Fig. 3).

If the fraction (r) of a total tissue dose-rate of neutron-γ mixed radiation is contributed by fission neutrons, the induced apoptosis rate by (n + γ) radiation ($b_{n+\gamma}$) can be approximated by

$$b_{n+\gamma} = (1 - r)b_\gamma + rb_n, \tag{4}$$

where b_γ is the induced apoptosis rate by γ rays and b_n the induced apoptosis rate by fission neutrons.

Since the b value of γ rays (b_γ) is equal, within experimental errors, to that of X rays (b_x)

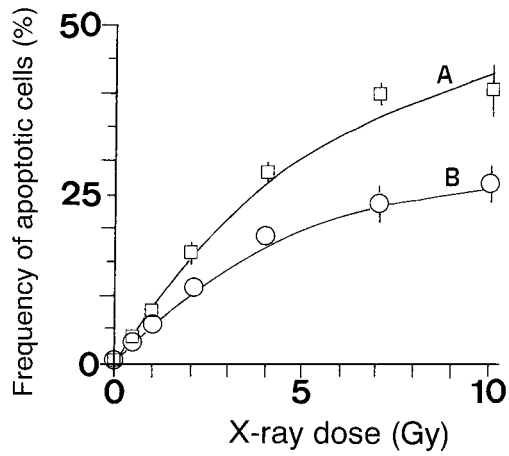

Fig. 2. Frequency of apoptotic cells (F) in the thymus plotted against X ray doses administered whole-body to mice of strain ICR. The mice were sacrificed 4 h after irradiation. **Curve A**: The F values were determined after the haematoxylin-eosin (HE) staining. Cells with pyknotic nuclei (see Fig. 1A) were scored as apoptotic cells. **Curve B**: The F values were determined after TUNEL staining.

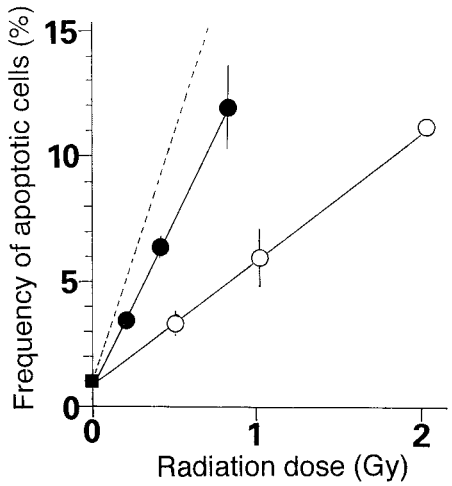

Fig. 3. Dose-response curves for the frequency of apoptotic cells in the thymus after the whole-body irradiation of mice with X rays (open circles) and with fission neutron-γ mixed radiation (closed circles). The mice were sacrificed 4 h after irradiation. The frequencies of apoptotic cells were determined by TUNEL staining. The broken line is a theoretical dose-response curve after exposure to fission neutrons.

as will be shown later (see Table 6 and its footnote), by substituting b_x for b_γ in Eq. 4 we obtain the following equation:

$$b_n = b_x + (b_{n+\gamma} - b_x)/r, \tag{5}$$

From the data used to construct Fig. 3, we have the values b_x and $b_{n+\gamma}$, as shown in Table 3. Substituting these numerical values for b_x and $b_{n+\gamma}$ in Eq. 5 and using the experimental r value of 0.5 (i.e., the ratio of 0.2 Gy/hr for fission neutrons to 0.4 Gy/hr for mixed radiation), we find from Eq. 5 that the theoretical value of b_n is 0.23 (see Table 3).

The broken straight line given in Fig. 3 is the theoretical response curve for the induction of apoptosis by fission neutrons based on the assumption that $b_n = 0.23$ (Gy^{-1}).

From the ratio of the b_n value to the b_x value based on TUNEL staining, the RBE of fission neutrons for the induction of apoptosis *in vivo* is 4.6 for the thymus and 4.2 for the spleen

Table 3. Induced rate per Gy of apoptotic cell deaths in thymus and spleen after the whole-body irradiation of ICR mice obtained with X rays (b_x), with fission neutron-γ mixed radiation ($b_{n+\gamma}$) and with fission neutrons (b_n)

Organ	Apoptosis detected by	b_x	$b_{n+\gamma}$	b_n	RBE of fission neutrons ($=b_n/b_x$)
Thymus	TUNEL staining	0.05 ± 0.01	0.14 ± 0.01	0.23 ± 0.02	4.6 ± 0.5
	HE staining	0.07 ± 0.01	0.14 ± 0.03	0.22 ± 0.06	3.1 ± 0.9
Spleen	TUNEL staining	0.05 ± 0.01	0.13 ± 0.01	0.21 ± 0.02	4.2 ± 0.6

(Table 3). However, for the apoptosis induction detected by HE staining, the fission neutrons' RBE is 3.1 for the thymus (Table 3).

Relative biological effectiveness of fission neutrons for causing atrophies in the thymus and spleen

Conspicuous atrophies in the thymus and spleen were observed 24 h after the whole-body X-irradiation of mice. Based on quantitative measurements, we found that the weight (M) of the thymus 24 h after irradiation decreased exponentially with the increase in the dose (D (Gy)) of X rays or fission neutron-g mixed radiation down to about 50% of the weight of the unirradiated thymus, M_o (Fig. 4). Namely, we obtained the following empirical relation between M and D:

$$M = M_o \exp(-mD) \text{ for } M/M_o \geq 0.5, \tag{6}$$

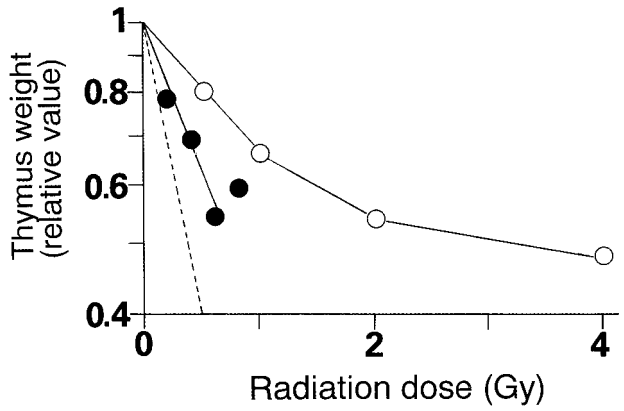

Fig. 4. Dose-response curves for the weight loss of the thymus after the whole-body irradiation of ICR mice with fission neutron-γ mixed radiation (closed circles) and with X rays (open circles). The broken line is a theoretical curve after exposure to fission neutrons. The mice were sacrificed 24 h after irradiation and the weight of the thymus was then measured.

Table 4. Empirical and theoretical values for the induced weight loss rate (Gy^{-1}) (m)[a] of the thymus and spleen by X rays, fission neutron-γ mixed radiation and fission neutrons

Organ	Induced weight loss rates per Gy			RBE of fission neutrons (m_n/m_X)
	m_X[b]	$m_{n+\gamma}$[b]	m_n[b]	
Thymus	0.43 ± 0.03	1.05 ± 0.16	1.67 ± 0.32	3.9 ± 0.8
Spleen	0.46 ± 0.05	1.04 ± 0.33	1.62 ± 0.66	3.5 ± 1.5

[a] m was calculated from the M versus D (Gy) relation $M = M_o \exp(-mD)$, where M is the weight of the thymus or spleen at dose D and M_o the M value of the unirradiated organ.

[b] m_X, $m_{n+\gamma}$ and m_n are the induced weight loss rate per Gy of X rays, fission neutron-γ mixed radiation and fission neutrons, respectively.

where m is the induced weight-loss rate per Gy. Applying Eq. 6 to the data points given in Fig. 4, we obtain the empirical values for m_X (induced weight loss rate per unit dose of X rays), and $m_{n+\gamma}$ (induced weight loss rate per unit dose of fission neutron-γ mixed radiation) and a theoretical value for m_n (induced weight loss rate per unit dose of of fission neutrons), as summarized in Table 4. The m_n value has been calculated from the m_X and $m_{n+\gamma}$ values in a way similar to a calculation of the b_n value in Table 3. The ratio of m_n to m_X gives an RBE value of 3.9 to fission neutrons for the induction of thymus atrophy (Table 4). Similarly, we find that for the induction of spleen atrophy, the RBE of fission neutrons is 3.5 (Table 4).

Evidence that radiation-induced apoptotic cell death is independent of the dose rate

The RBE of 4.6 of fission neutrons for the induction of apoptosis given in Table 3 was obtained from the ratio of b_n to b_x. However, the dose-rate of the neutron-γ mixed radiation was one hundred-times lower than that of X rays. Therefore, the validity of 4.6 for the RBE of fission neutrons depends on whether the induced apoptosis rate by X or γ rays is independent of the dose rate. In order to examine this problem, the following experiments were carried out.

Mice were exposed to various doses of X or γ rays at high dose rates (0.3 or 0.4 Gy/min) or at a low dose-rate (3 mGy/min). They were sacrificed 24 h after irradiation and the thymuses and spleens were then removed. Lymphocytes released from each organ were stained in Tuerk's solution and cells stained 'violet' were scored as surviving lymphocytes. The results are summarized in Table 5. As can be seen from the table, the survival (S) of lymphocytes decreased approximately exponentially with the increase in the dose following Eq. 3. Applying Eq. 3 to the S values given in Table 5, we obtained k values at different dose-rates, as

Table 5. Changes in the number or fraction (S) of surviving lymphocytes in thymus and spleen of ICR mice 24 h after irradiation with various doses of X or γ rays at different dose rates

Radiation	Dose rate (Gy/min)	Dose (Gy)	Number of lymphocytes and its relative ratio S in			
			Thymus		Spleen	
			No. ($\times 10^6$ cells)	S	No. ($\times 10^6$ cells)	S
Control	0	0	235 ± 94	1	218 ± 58	1
X rays	0.3	0.5	116 ± 23	0.49	131 ± 24	0.60
		1	77 ± 13	0.33	101 ± 13	0.46
		2	40 ± 10	0.17	71 ± 27	0.33
		4	13 ± 7	0.06	23 ± 10	0.11
^{60}Co γ rays	0.4	1	73 ± 9	0.31	99 ± 18	0.45
		2	27 ± 10	0.11	54 ± 14	0.25
		4	20 ± 10	0.09	20 ± 3	0.09
Fission	0.007	0.2	119 ± 17	0.51	140 ± 28	0.64
neutron-γ		0.4	110 ± 55	0.47	118 ± 5	0.54
mixed		0.6	60 ± 14	0.26	96 ± 9	0.44
radiation		0.8	53 ± 17	0.23	88 ± 16	0.40
^{137}Cs γ	0.003	0.5	130 ± 45	0.55	117 ± 32	0.54
rays		1	80 ± 26	0.34	85 ± 5	0.39

Table 6. Changes in the radiation-induced reduction rate k (Gy^{-1}) of surviving lymphocytes in the thymus and spleen measured 24 h after the irradiation of ICR mice with X or γ rays at different dose rates

X or γ rays	Dose rate	Induced reduction rate k (Gy^{-1})[a] in surviving lymphocytes in Thymus	Spleen
X rays	0.3 Gy/min	1.14 ± 0.27	0.78 ± 0.23
γ rays	0.4 Gy/min	1.13 ± 0.05	0.74 ± 0.06
γ rays	0.003 Gy/min	1.13 ± 0.08	1.09 ± 0.20

[a] k was calculated from the data of survival S (of lymphocytes) versus D (Gy) relation at D = 0.5, 1 and 2 Gy given in Table 5, using the empirical equation $S = \exp(-kD)$. Since k is the total inactivation rate per Gy of lymphocytes by apoptosis in 24 h after irradiation, the observed result that the k value of X rays (k_x) is equal to that of γ rays (k_γ) means that lymphocyte apoptosis rate per Gy of X rays (b_x) is equal to that of γ rays (b_γ).

shown in Table 6. As can be seen in Table 6, the k values are equal within the experimental errors, not only between X and γ irradiations, but also between high dose-rate (0.4 Gy/min) and low dose-rate (0.003 Gy/min) γ-irradiations.

DISCUSSION

The rate b (Gy^{-1}) of induced apoptotic cell frequency 4 h after X-irradiation is 0.18 and 0.14 , respectively, for the thymus and spleen of the p53(+/+) C57BL/6 strain (Tables 2). Since the relative ratios $b : m : k$, where m (Gy^{-1}) is the weight-loss rate and k (Gy^{-1}) the lymphocyte content-loss rate 24 h after X-irradiation for the thymus or spleen, are about 1:3:4 (see Rrs of Table 2), after the X-irradiation of p53(+/+) mice, the frequency of lymphocytes in the thymus or spleen dying by apoptosis 4 h after irradiation is about one third to one fourth of the total fraction of the lymphocytes lost by apoptosis 24 h after irradiation. This conclusion is compatible with the fact that the peak of radiation-induced apoptosis in the thymus is very sharp around 4 h after irradiation *in vivo*[6,7,18,19]. Previously, Surh and Sprent[7] reported that compared with the unirradiated thymus of C57BL/6 mice, the cell yields from 1 Gy-irradiated thymuses were reduced by 15% 2 h after irradiation, 30% after 4 h and 68% after 24 h, indicating that a 15% loss of lymphocytes by apoptosis occurs during the period 2 to 4 h after irradiation, accounting for one quarter of the total loss of lymphocytes measured after 24 h. These and the present experiments demonstrate that the loss in the weight or the lymphocyte content of the thymus 24 h after irradiation is a simple, reliable measure of the cumulative effects of cells dying by apoptosis in a complicated process which depends on the time after irradiation and the site in the thymus[7,18]. The atrophic changes in the spleen observed 24 h after irradiation could serve as an equally good measure of the total apoptotic cell death (see Tables 1 and 2).

Unexpectedly, the whole-body irradiation of p53(–/–) mice produced hyperplastic effects in their thymus and spleen, as can be seen in Table 1; the increase in the organ weight was about 10 and 6% for the thymus and the spleen, respectively, whereas the increase in the lym-

phocyte content was 73 and 33% for the thymus and spleen, respectively (Table 1). This radiation-inducible mitotic activity existent in p53 (–/–) mice may be related to the tumorigenesis-prone characteristics of p53(–/–) mice[20,21]. Furthermore, the irradiation of p53(–/–) mice revealed that there was a small, but significant increase, in the apoptosis frequency in the spleen but not so in the thymus when measured 4 h after irradiation, whereas in the thymus of p53(–/–) mice there was a significant increase in the apoptosis frequency 72 h after irradiation, but no increase after 4 h (Table 1). These intriguing findings support the recent proposal by Khwaja[22] that "arm chair-based generalizations on how apoptosis is controlled are no longer tenable; the focus may need to shift away from cell lines to primary tissues".

The RBE value of fission neutrons for apoptosis induction in thymocytes detected by TUNEL straining was 4.6 ± 0.5 (Table 3). This RBE value agrees, within the experimental errors, with our previously obtained RBE value, which was 5.1 ± 0.3, of the same fission neutrons for the induction of crossing-over mutations in *D. melanogaster*[13]. These mutations resulted from recombinational or gene conversion repair of double-strand breaks (DSBs) in DNA. This agreement supports the notion that the primary cause of radiation-induced apoptosis is mostly DSBs. However, the RBE of fission neutrons for apoptosis detected by scoring cells with pyknotic nuclei after HE staining was 3.1 ± 0.9, which is considerably, though not significantly, smaller than the RBE of fission neutrons for apoptosis induction detected by TUNEL staining (Table 3). This discrepancy may suggest that apoptotic cells detected for pyknotic nuclei after HE staining were partly not identical to those detected by TUNEL staining for existence of fragmented DNA. In support of this interpretation, Nakagawa et al[23] reported that the introduction of blunt-ended DSBs by microinjection of a specific restriction enzyme into normal human fibroblasts resulted in nuclear accumulation of p53 protein due to phosphorylation at serine 15, whereas p53 accumulation was not observed in similarly treated ATM-defective (AT) fibroblasts; moreover, when AT fibroblasts were treated with X rays, a small, but significant, fraction of cells showed phosphorylation at serine 15. Zhao et al[24] reported that when Molt-4 human leukemia cells were exposed to X rays, cells showed two types of apoptotic responses: one was dependent on the induction of p53's downstream gene Bax; the other was mediated by an enhancement of the intracellular free-calcium ions (Ca^{2+}). These reports are compatible with the assumption that the primary cause of radiation-induced apoptosis is DSBs with a minor role played by other types of damage produced by reactive oxygen species or other toxins.

After whole-body exposure to dose D of γ rays at a high dose-rate (0.4 Gy/min) or a low dose-rate (3 mGy/min), the surviving fractions (S) of lymphocytes in the thymus or spleen 24 h after irradiation changed with D following the equation $S = \exp(-kD)$. We experimentally determined the k value from the observed S values for the two organs (Table 5); thus, the obtained k values are the same for irradiations at the high and low dose-rates (see Table 6), showing that radiation-induced apoptosis is independent of the dose rate.

In contrast, major risks of low-level radiation, i.e., mutagenesis, teratogenesis and carcinogenesis, are dose-rate dependent events. All of the three have been demonstrated experimentally with mice to approach zero risk by lowering the dose rate (review by Kondo[25]). This shows the critical contribution of DNA repair, especially recombinational or end-joining

repair[25] of DSBs in prevention of radiation risk. However, DNA repair is not perfect. There must be other defense mechanisms against DNA damage. Apoptosis has emerged as another component in the defense mechanisms. There now appears to be another facet of the apoptotic elimination of cells with unrepaired DNA damage that occurs independently of the radiation dose-rate as observed in the present experiments. This implies that for complete elimination of DNA damage from irradiated tissues in cooperation with DNA repair, the complementing role of apoptosis becomes more effective during chronic irradiation when DSBs produced in irradiated DNA are proficiently dealt with by the slow process of DNA repair (see below) than during acute irradiation when DSBs are produced in too short a time to be dealt with by slow repair. This supports the hypothesis recently proposed by Kondo[25], who predicted that radiation-induced damage is completely eliminated from tissues if there is concerted cooperation between DNA repair and apoptosis.

A simple hypothesis concerning dose-rate independence of apoptosis after irradiation is as follows. In lymphocytes, apoptosis occurs so rapidly after the production of DSBs by radiation that most cells with DSBs undergo apoptosis before the DSBs are repaired. The recombinational repair of DSBs in mammalian cells occurs only after cells with DSBs enter the S phase, because the Rad51 protein, indispensable for this repair, is synthesized in the S-G2 phases and degraded in the M phase[27]. If the cell apoptosis is slow, only a fraction of the cells undergo dose-rate independent apoptosis. This is the case for apoptosis after irradiation in spermatogonia (unpublished).

The last, but not the least, important question is why do lymphocytes show vigorous p53-dependent apoptosis activity after exposure to even a small dose of ionizing radiation? Guidos et al[28] proposed the hypothesis that p53 is operative in the detection and management of DSBs that arise during the process of V(D)J recombination. They presented the following results to support this hypothesis. In p53(–/–) scid mice, the severe thymus atrophy of *scid* mice was considerably rescued by nullification of the p53 gene, as evidenced by a slow, but steady, increase in the cellularity of the thymus from 2×10^6 cells at an age of ten days to 10×10^6 cells after 5 weeks. This recovering effect was partly due to the prolonged survival of lymphocytes in the double-mutant mice that allowed the accumulation of lymphocyte precursors, especially a dramatic accumulation of pro-B cells in the thymus. A considerable fraction of these immature cells were aneuploid. All of the p53(–/–) scid mice developed disseminated pro-B or immature T cell lymphoma or leukemia. These results demonstrate that p53-dependent vigorous apoptosis activity is indispensable not only for *scid* mice, but also for normal mice, to suppress the oncogenic risk of DSBs produced during V(D)J recombination.

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