Programmed Cell Death in Whole Body and Organ Systems by Low Dose Radiation

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Programmed cell death/Whole body/Organ culture/Low dose radiation/Mouse

New whole-body and organ systems were established to detect interphase cell death in the thymus, spleen and epithelial cells of intestinal crypts by low-dose radiation. Frozen sections of the thymus, spleen and intestine as thick as 8 μm were made after X-irradiation of whole body or removed organs, and then sections were stained with 0.02% erythrosin B solution. In unirradiated controls, a few numbers of erythrosin B positive cells (dead or dying cells) were observed in the thymus, spleen and intestinal crypt as a single cell death. When X rays were given to various strains of mice as a whole body dose, clusters of erythrosin B positive cells were produced. They appear at 2 hr after irradiation and reached maximum at 4 hr, remaining at a similar level until 8 hr after irradiation. The number of erythrosin B positive cells decreased after then by the elimination of dead cells, and they were observed like a single cell death at 24 hr after irradiation. When erythrosin B positive cells were scored 4 hr after irradiation, their total number and the number of cluster increased with increasing doses of X rays in the dose range from 0.05 to 0.5 Gy. It is noted that there were large differences in the radiation susceptibility among the inbred strains of mice for the induction of interphase cell death of thymic lymphocytes: e.g., high susceptibility in C57BL/6J and AKR/J, intermediate in N4, A/J, PT and ST, low in C3H/HeJ, HT, 101/H and DBA/2J, indicating that interphase cell death is genetically programmed. Similar results were observed with some chemical mutagens. Although a large increase of erythrosin B positive cells was observed in the thymus and spleen with methylprednisolone, there was no increase in the intestinal crypt, and vice versa with bleomycin, suggesting the organ specificity for the induction of interphase cell death by chemicals.

For the in vitro method, the removed thymus was irradiated on the agar plate, and then incubated on the agar plate which was placed on the grid in the medium, so that the medium comes up to the organ through the agar plate. Frozen sections were made and stained with erythrosin B solution in the same way as the in vivo method. The number of erythrosin B positive cells in the organ culture system reached maximum at 5 hr after X-irradiation, e.g. slightly later than in the whole-body system. The efficiency was about 60% in C57BL/6J mice when compared with whole-body system.

INTRODUCTION

Radiation is known to induce two types of cell deaths. One is closely related to cell division and induced frequently by the radiation exposure (considerably high doses) at early S

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and M stages. Since the cell dies during or after cell division, sometimes after several
generations, or looses its reproductive activity, this type of death is called as division death,
reproductive death, or mitotic death\(^1\). If cells are not killed by radiation, it causes mutation,
cancer, malformation, etc. in the survived cells and tissues.

The other type of death is induced by radiation in the cell at the interphase stage or in the
terminally differentiated cells. Since this type of death is induced very shortly after irradiation
with the characteristic nuclear and cytoplasmic changes, or spontaneously occur during the
embryonic development and the aging, interphase cell death is called by the term, suicide death,
greek name apoptosis, or programmed cell death\(^1\)\(^-\)\(^9\). Interphase cell death has been studied
mainly in the lymphatic organs (thymus and spleen) and epithelial cells of the intestinal crypt
and glandular organs by measuring picnotic cells or survived cells\(^1\)\(^-\)\(^9\). Interphase cell death has
been also studied with isolated thymic cells and cultured tumor cells\(^6\)\(^\)\(^7\). In these \textit{in vitro}
studies, however, cell death is induced by the considerably high doses (5 to 10 Gy), while very
small doses of radiation induce it in the \textit{in vivo} system.

In this study, we established an extremely sensitive \textit{in vivo} method to detect interphase cell
death in the thymus, spleen and intestinal crypt with the use of frozen section of these organs
and tissues. By this method, we examined dose-response relationship with low doses of
radiation and some chemicals, \textit{e.g.} glucocorticoid which is known to induce apoptosis\(^8\), and
results were compared with usual methods. Moreover, organ culture of the thymus was carried
out to detect interphase cell death.

\textbf{MATERIALS AND METHODS}

\textit{Mice.}

Mouse strains used in this experiment were; C57BL/6J (F\(_{129+30}\)), ST/bJ (F\(_{123+25}\)), A/
J(F\(_{172+17}\)), C3H/HeJ (F\(_{157+30}\)), RF/J (F\(_{118+4}\)), AKR/J (F\(_{164+4}\)), DBA/2J (F\(_{158+7}\)), 101/H
(F\(_{80+19}\)), PT (F\(_{25}\)), HT (F\(_{13}\)) and ICR/N4 (F\(_{20+35}\)). Pedigree identified pairs of the first 4
strains were provided by Dr. E. S. Russell (The Jackson Laboratory, Bar Harbor, ME, U.S.A.)
in 1978 and the subsequent 3 pedigree identified strains were provided by the Jackson
Laboratory in 1985. These strains of mice were maintained by brother\(\times\)sister inbreeding by
Dr. E. S. Russell and staffs of the Jackson Laboratory and then by T. Nomura in the
Department of Radiation Biology, Faculty of Medicine, Osaka University. Inbred 101/H and
outbred PT and HT strains were provided by Drs. M. F. Lyon and A. G. Searle (Radiobiology
Unit, Medical Research Council, Harwell, U.K.) in 1978 and then they are maintained by b\(\times\)s
inbreeding by T. Nomura in the Department of Radiation Biology\(^9\). ICR/N4 (N4) is a new
inbred strain established by T. Nomura from the ICR stock of Dr. I. Taki. All of these mouse
strains were maintained in the complete barrier condition with mouse diet CRF-1 (Charles River
Japan, Kanagawa, Japan) and chlorinated water at 23\(\pm\)1°C.

\textit{X-ray Exposure.}

A Shimazu Model SHT-250 M3 X-ray apparatus (Shimazu, Kyoto, Japan) was used for
X-irradiation at the distance of 51.5 or 100.5 cm from the center, operating at 20 mA and 180 KVP with a filter of 0.5 mm of copper and 1.0 mm of aluminum. Doses of X rays were measured at each time by a Victoreen Model 570 r-Meter (Victoreen, Instrument Division, Cleveland, OH, U.S.A.) which was adjusted by Fricke dosimetry. Average dose rate was 0.507 ±0.006 Gy/min (mean ±95% CL, n=159) at total doses of 0.1 Gy and more, and 0.135±0.024 Gy/min (n=10) at a dose of 0.05 Gy.

Chemicals.

Methylprednisolone (Upjohn Co., Crawly, U.K.) and bleomycin (Nippon-Kayaku Co., Tokyo, Japan) were dissolved in 0.9% sterile NaCl solution just before use at concentrations of 250, 500, 1000 and 2000,ug/ml for methylprednisolone and 250, 1000 and 5000,ug/ml for bleomycin.

Measurement of Survived Cells.

A single whole body dose of X rays was given to N4 and C3H/HeJ mice. Mice were sacrificed 0, 2, 4, 6, 12, and 24 hr after irradiation, and then the thymus and spleen were removed and put into the petri dish (r=30 mm, Becton Dickinson & Co., CA, U.S.A.) containing the 10 ml of Hanks’ solution (pH 7.4, Nissui Pharmaceutical Co., Tokyo, Japan) immediately after killing. The thymus was gently pressed by forceps to release all thymic lymphocytes until only the capsule remains. Splenic cells were released by the gentle injection of 10 ml of Hanks’ solution into the spleen by the syringe with 26G needle. Released cells were stained with Türk’s solution (Kishida Chemical Ind., Osaka, Japan) and then nucleated cells were counted by Biirker-Turk hemacytometer.

To distinguish the T and B cells, thymic and splenic cells of C3H/HeJ mice were stained with ethidium bromide (Sigma Chemical Co., St. Louis, MO, U.S.A.), and then surviving T and B cells were stained with monoclonal anti-Thy-1.2 (New England Nuclear, Boston, MA, U.S.A.) and anti-mouse IgG (Cappel, West Chester, PA, U.S.A.) conjugated with fluorescein, respectively.

Direct Detection of Cell Death in Frozen Section.

Immediately after the killing of irradiated or unirradiated mice, the thymus, spleen and terminal ileum were removed gently and embedded into the O.C.T. compound (Miles Inc., Elkhart, IN, U.S.A.). The embedded organs were placed on the steel plate cooled at -20°C for more than 15 min. The frozen section was made by the cryostat (Miles Inc.) as thick as 8 μm and put on the slide glass. The frozen sections on the slide glass were stained with 0.02% erythrosin B solution (Nacalai Tesque Co., Kyoto, Japan, dissolved in Hanks’ solution) for 30 sec at 25°C. Dead or dying cells were stained in red with erythrosin B, because they can not excrete incorporated dye. Stained sections were covered by 24×32 mm cover glass and sealed with nail polish enamel. The number of erythrosin B positive cells was counted by the microscope (Fig. 1).
Organ Culture.

The thymus and spleen of C57BL/6J and C3H/HeJ mice were removed and put into the petri dish (\( r = 30 \text{ mm}, \) Becton Dickinson) which was placed on the warm plate at 37°C and in the
5% CO₂ air. Petri dish was precoated with 1% agar dissolved in the culture medium as thick as 3 mm. The organs in the above condition were irradiated or unirradiated, and then incubated on the agar plate (1% agr in the medium, 3 mm thick 10 mm wide 45 mm long) which was placed on the stainless steel grid in the petri dish. Both edges of the agar plate were soaked in the medium so that the medium came up to the organ through the agar plate (Fig. 2). The organs were incubated at 37°C in the 5% CO₂ air. The culture medium used in this experiment is the same as that used for the cultivation of the genital tract. The frozen sections were made 0, 2, 3, 4, 5, 6, 7, 8 hr after irradiation and stained as described above.

Fig. 2. Method to cultivate thymus for the detection of interphase cell death. Experimental procedures are given in the Materials and Methods.

Measurement of Erythrosin B Positive Cells.

The number of erythrosin B positive cells in the square field of 250 x 250 μm² was counted by the microscope at the magnification of 400. The area counted for the erythrosin B positive cells was 250-500 μm depth from the surface of the thymus cortex. In the cultured organs, erythrosin B positive cells in the area just beneath the surface was counted, because gas and medium more easily reach the surface area than the central area of the organ. For the splenic cells, erythrosin B positive cells were counted in the middle area of the follicle. Only the villi which were longitudinally sectioned were submitted to the measurement of erythrosin B positive cells in the crypt. Erythrosin B positive cells were counted in the 20 fields of the thymus cortex and splenic follicles and 30 to 40 crypts in each animal.

Each mouse (or organ) is numbered in the mouse facility of the Department of Radiation Biology. Only the mouse (or organ) No. is recorded when the mouse is irradiated or submitted to microscopic examination. After the measurement of the number of erythrosin B positive cells in each mouse (organ), data were classified into the experimental or dose groups. Consequently, blind experiments have been automatically carried out in our Department.
RESULTS

Survival of Thymic and Splenic Cells and Peripheral White Blood Cells.

As shown in Fig. 3, the number of thymic and splenic cells increased temporally at 2 hr after whole-body irradiation and decreased rapidly after then. Survival rate reached less than 5% of unirradiated controls in 24 hr, when 2.5 Gy of X rays were given to N4 mice. More rapid decrease was observed in the peripheral white blood cells.

Fig. 4 indicates dose-response curve. Survival rate of the thymic and splenic lymphocytes decreased with increasing doses of X rays. Survival rate of peripheral white blood cells also showed similar pattern. T cells were slightly more sensitive to radiation killing than B cells (Fig. 5).

Time Course of Interphase Cell Death after Whole-body Radiation.

The microscopic view of frozen sections of the thymus and intestinal crypt stained with erythrosin B were shown in Fig. 6a-f. There were a few cells stained in red and all are singly stained in unirradiated mice and also just after whole body exposure to 2.5 Gy of X rays. There was cytoplasmic fragmentation. Although there were no increase of erythrosin B

Fig. 3. Survival of thymic and splenic cells and peripheral white blood cells after the whole body dose of X rays. Adult N4 mice (3 to 4 months old) were exposed to a whole body dose of X rays (2.5 Gy), and then they were killed 0, 2, 4, 6, 12 and 24 hr after X-irradiation. Details for the measurement of thymic and splenic cells and peripheral white blood cells are given in the Materials and Methods. Four mice were killed at each time. •; thymus, ○; spleen, △; peripheral white blood cell.
Fig. 4. Relative survival of thymic and splenic cells and peripheral white blood cells after whole body doses of X rays. Adult N4 (a) and C3H/HeJ (b) mice (3 to 4 months old) were exposed to 0, 0.5, 1.0 and 2.5 Gy of X rays, and then killed at 24 hr after X-irradiation. Details for the measurement of thymic and splenic cells and peripheral white blood cells are given in the text. Four N4 and C3H/HeJ mice were examined at each dose. •; thymus, ○; spleen, △; peripheral white blood cell.

Fig. 5. Relative survival of T and B cells in the thymus (a) and spleen (b) after whole body doses of X rays. Adult C3H/HeJ mice were exposed to 0, 0.5, 1.0 and 2.5 Gy of X rays and then killed at 24 hr after X-irradiation. Survived cells were stained with monoclonal antibodies conjugated with fluorescein to identify T and B cells. Two mice were examined at each dose. •; T cell, ○; B cell.
Fig. 6. Microscopic view of interphase cell death in the thymus (a-d, f) and intestinal crypt (e) of N4 mice stained with erythrosin B. ×360. (a) unirradiated control, thymus, (b) 2 hr after irradiation (2.5 Gy), thymus, (c) 4 hr after irradiation (2.5 Gy), thymus, (d) 24 hr after irradiation (2.5 Gy), thymus, (e) 4 hr after irradiation (2.5 Gy), intestinal crypt, (f) 4 hr after irradiation (0.05 Gy), thymus.
positive cells 1 hr after irradiation, erythrosin B positive cells form the cluster 2 hr after irradiation (Fig. 6b). The size and number of clusters reached maximum at 4 to 6 hr after irradiation (Fig. 6c). After then, however, dead cells were eliminated and sized of clusters became smaller. In 24 hr, several erythrosin B positive cells remained and looked like a single cell death (Fig. 6d). Similar views were observed in the frozen section of the spleen. Thus, clusters of cell deaths were induced in the lymphatic apparatus at early stages after X-irradiation, although they disappear by the elimination of dead cells and small numbers of dead and living cells remained in 24 hr (Figs. 3, 4 and 6d). Superficially it looks like a single cell death. Similar erythrosin B positive cells were detected in the intestinal crypt shortly after X-irradiation (Fig. 6e).

![Graphs showing erythrosin B positive cell counts and cluster sizes](image)

**Fig. 7.** Erythrosin B positive cells in the thymus, spleen and intestinal crypt after whole body doses of X rays. Adult N4 mice (3 to 4 months old) were exposed to 0, 0.05, 0.1, 0.25 and 0.5 Gy of X rays and then killed 4 hr after X-irradiation. The total number of erythrosin B positive cells in the thymus cortex (a), spleen follicle (b) and intestinal crypt (c), the number of cluster (d) (the number of colonies of erythrosin B positive cells), and the number of singly stained cells (f) per field (250 μm²) and the size of cluster (e) (the number of erythrosin B positive cells per colony) were given on the ordinate against treated doses of X rays. The number of mice examined was 10, 9, 10, 11 and 20 at doses of 0, 0.05, 0.1, 0.25 and 0.5 Gy, respectively. Vertical bars indicate 95% confidence intervals of the mean computed from the t-distribution of the mean.
Dose Response and Strain Differences

Fig. 7 shows another characteristic of interphase cell death. When X rays were given to N4 mice and erythrosin B positive cells were measured 4 hr after irradiation, the number of erythrosin B positive cells increased almost linearly in the thymus and spleen in the dose range from 0.05 to 0.5 Gy. Clusters of erythrosin B positive cells were detected with 0.05 Gy and more doses (Fig. 6c, f). Large increase of erythrosin B positive cells was also observed in the intestinal crypt. Not only the total number of erythrosin B positive cells but also the number of cluster of erythrosin B positive cells increased greatly with doses of X rays (Fig. 7d), while such an increase was not consistent for singly stained cells (Fig. 7f) and the size of cluster was within only 1.6 fold of the control at the maximum dose (Fig. 7e).

Fig. 8 shows dose response curves of X-ray induced erythrosin B positive cells in the thymus, spleen, and intestinal crypt of various strains of inbred mice. Erythrosin B positive cells increased almost linearly in the thymus, spleen and intestinal crypt with increasing doses of X rays in the dose range from 0.05 to 0.5 Gy. It is noted that there were large differences in the number of X-ray induced interphase cell death among mouse strains. C57BL/6J and AKR/J showed high susceptibility, N4, RF/J, A/J, PT and ST showed intermediate susceptibility, and C3H/HeJ, HT, 101/H and DBA/2J were resistant, indicating that the susceptibility to X-ray induced interphase cell death is genetically determined.

Fig. 8. Erythrosin B positive cells in the thymus, spleen and intestinal crypt of the various strains of inbred mice after whole body doses of X rays. Adult C57BL/6J (●), C3H/HeJ (○), AKR/J (▲), HT (△), RF/J (■), DBA/2J (□), A/J (▼), 101/H (▲), PT (●) and ST/bJ (○) mice (3 to 4 months old) were exposed to 0, 0.1, 0.25 and 0.5 Gy of X rays and then killed at 4 hr after X-irradiation. The total number of erythrosin B positive cells in the thymus cortex (a), spleen follicle (b) and intestinal crypt (c) was given on the ordinate. The number of mice examined was 40, 36, 24, 20, 22, 20, 14, 12, 17 and 16 for the strains described above, respectively.
Similar results were observed in the splenic cells and epithelial cells of intestinal crypt, but with different susceptibility from the thymus.

**Cell Death by Chemicals.**

Not only radiations but also chemicals induced interphase cell death (Fig. 9). Small amounts of methylprednisolone produced erythrosin B positive cells in the thymus and spleen with a variety of susceptibility among mouse strains as in the case of X rays. A high susceptible strain to X rays was also susceptible to this chemical, and *vice versa*. However, there was no increase of erythrosin B positive cells in the intestinal crypt after methylprednisolone treatment. Oppose result was observed with bleomycin. There was a small increase of erythrosin B positive cells in the thymus and spleen but a dramatic increase in the intestinal crypt. Consequently, there is a tissue specificity in addition to genetic predisposition for the susceptibility to interphase cell death.

![Graph](image)

**Dose of methylprednisolone and bleomycin (µg/g)**

Fig. 9. Erythrosin B positive cells in the thymus, spleen and intestinal crypt in N4 mice after treatment with methylprednisolone and bleomycin. Methylprednisolone or bleomycin was injected intraperitoneally into adult N4 mice (3 to 4 months old), and then mice were killed at 4 hr after injection. Measurement of erythrosin B positive cells was given in the text and legends to Fig. 8. The number of mice examined was 10, 7, 8, 14 and 8 at doses of 0, 2.5, 5, 10 and 20 µg per g of body weight of methylprednisolone, and 10, 10, 7 and 8 at doses of 0, 2.5, 10, and 50 µg per g of body weight of bleomycin. ○, △ and ■ for the thymus, spleen and intestinal crypt of mice treated with methylprednisolone, ○, △ and □ for those treated with bleomycin, respectively. Vertical bars indicate 95% confidence intervals of the mean computed from *t*-distribution of the mean.

**Detection of Cell Death in the Cultured Organ.**

When 0.5 Gy of X rays were given to the removed thymus of C57BL/6J and C3H/HeJ mice, and then the organ was incubated *in vitro*. The number of erythrosin B positive cells
increased 3 hr after irradiation, and reached maximum at about 5 hr after irradiation, i.e., slightly later than the whole-body system (Fig. 10a).

Fig. 10b shows the dose-response curves of erythrosin B positive cells. X rays were given to the thymus removed from C57BL/6J and C3H/HeJ mice and incubated on the agar plate for 5 hr. The number of erythrosin B positive cells increased with doses of X rays in both strains. Efficiency was about 60% in C57BL/6J mice when compared with whole body system. There remain some improvements, but the number of erythrosin B positive cells detected in the cultured thymus of C3H/HeJ mice was almost equal to that in the whole body system.

Temperature Dependency of Programmed Cell Death.

The organ was irradiated with 0.5 Gy of X rays and then incubated for 5 hr at the temperature of 4, 15, 25 and 37°C to examine the involvement of enzymatic pathway in the process of cell death. Only at 37°C, interphase cell death was induced by X rays. When the thymus was incubated at 4°C for 5 hr and then incubated for 4 hr at 37°C, moreover, interphase cell death was detected, although the number was smaller than that kept all times at 37°C. These results indicate that enzymatic processes are involved to induce interphase cell death.

![Graph showing Erythrosin B positive cells versus hours after exposure and dose of X rays.](image_url)
DISCUSSION

We have established a new method to detect interphase cell death in the more physiological condition by using the frozen section of the thymus, spleen and intestinal crypt. Actually, there should be no differences in the number of dead cells between the new method using frozen section and usual method collecting survived cells. Since small numbers of dead cells in the large population are directly counted in the present method, however, cell death is more effectively measured than the usual method by which the survived cells are compared. We believe this is the most sensitive method so far tested to detect the effect of low dose radiation. Large strain differences of the susceptibility in X-ray induced interphase cell death among mouse strains suggest that genetic predisposition is involved in the process of cell death. In the recent study, such a high susceptibility inherited as a dominant trait (Nomura, T. unpublished data), indicating that interphase cell death or apoptosis is a genetically programmed phenomenon.

By this quick method, furthermore, we could find early changes of interphase cell death and the location where cell death occurs in that organ. Classical criteria of interphase cell death or apoptosis was a single cell death (1-3). In the present study, however, we found that erythrosin B positive cells formed clusters in the thymus and spleen very shortly after irradiation even with a dose as low as 0.05 Gy (Fig. 7f), while in 24 hr most of dead cell were eliminated and
erythrosin B positive cells remained as if they were singly dead (Fig. 7d). Why X rays and chemicals form the cluster of cell death in the thymus and spleen? There might be a sensitive population of thymic and splenic lymphocytes. Alternatively, death of a radiation-sensitive cell induces subsequent deaths of surrounding lymphocytes through cell to cell communication like gap-junction. In fact, there were no substantial differences (only 1.6 times differences) in the average size of clusters between irradiated and unirradiated mice, while there were 10 times differences in the number of clusters (Fig. 7d, e). Temperature sensitivity for the induction of cell death indicates the involvement of biochemical or enzymatic processes in the cell killing. This finding is supported by the fact that cycloheximide and actinomycin D, inhibitor of protein or messenger RNA synthesis, respectively, inhibited radiation-induced cell death (Hongyo, T. and Nomura, T., unpublished data). Low dose radiation evokes genetically programmed processes to produce some compounds, and these compounds produced in a cell might transmit to surrounding cells by cell to cell communication.

What is the biological significance of the high susceptibility? One might be related to high susceptibility to X-ray induced leukemia, since high susceptible strains C57BL/6J and AKR/J were very sensitive to leukemia induction in that organ, while low susceptible strains C3H/HeJ and HT to interphase cell death were resistant to leukemia induction by X rays. We can predict leukemia susceptible strains of mice by this method, and may predict high susceptible human individual by in vitro method in future.

ACKNOWLEDGEMENT

The authors thank S. Kida, Y. Murakami, M. Kaji and C. Hisamatsu for their technical assistance and M. Maeda for typing the manuscript. The work is supported by the grants from the Japanese Agency of Technology and Ministry of Education, Science and Culture.

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