Apoptosis in the Chicken Bursa of Fabricius Induced by X-Irradiation

Soichiro ARAI, Tomomi KOWADA, Kazushige TAKEHANA¹⁾, Kunie MIYOSHI, Yuh H. NAKANISHI, and Masanobu HAYASHI

Departments of Veterinary Radiology and ¹⁾Veterinary Anatomy, Rakuno Gakuen University, Ebetsu 069, Japan

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ABSTRACT. Immature B lymphocytes in the chicken bursa of Fabricius have previously been reported to undergo apoptosis by low doses of ionizing radiation. In the present study, newly hatched chickens were subjected to whole-body X-irradiation, and the bursa of Fabricius was examined at various postirradiation times by light and electron microscopy to obtain information on the change of ultrastructure of irradiated bursal cells as well as on the time course and dose-response for the induction of apoptosis. Histological examination by light microscopy showed that pyknotic cells started to increase in the bursa within a few hours after irradiation and the frequency of occurrence reached a maximum at 6 hr. An evident increase of the pyknotic cells in number was observed at a dose of as little as 1 Gy, and the frequency increased with increases in the dose, reaching over 90% at 15 Gy. Electron microscopy of the irradiated bursa revealed typical apoptotic morphology such as chromatin condensation, nuclear fragmentation, formation of apoptotic bodies and phagocytosis of pyknotic cells. Induction of apoptosis was also confirmed by the appearance of a typical DNA ladder pattern on agarose gel. Thus, the present results demonstrate that the chicken bursal cells are hypersensitive to X-irradiation with regard to induction of apoptosis, and that the apoptotic bursal cells exhibit most of the ultrastructural features known to be typical of apoptosis. — **KEY WORDS:** apoptosis, bursa of Fabricius, chicken, electron microscopy, X-(ray)-irradiation.

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Lymphoid tissues such as the thymus, spleen and lymph nodes are known to be extremely sensitive to ionizing radiation [reviewed in 11]. The high radiosensitivity of the lymphoid tissues is mainly attributed to the radiation-induced death of lymphoid cells, which is caused by low-dose irradiation and usually observed within a few hours after irradiation. Extensive studies on radiation-induced death of mammalian thymocytes both *in vivo* and *in vitro* have shown that irradiated thymocytes die by apoptosis [13, 20]. Apoptosis is now recognized as a mode of cell death in which a cell suicide program is put into operation in response to a diversity of physiological or pathological stimuli [2, 8, 19]. This mode of cell death is characterized morphologically by cell shrinkage, chromatin condensation, membrane blebbing and the formation of apoptotic bodies, and biochemically by internucleosomal fragmentation of DNA [18].

The bursa of Fabricius is a primary lymphoid organ restricted to avian species, and lymphoid cells in this organ are composed largely of developing immature B lymphocytes. The organ, like other lymphoid organs, has been known to be highly sensitive to ionizing radiation [10, 17]. It has previously been reported that immature B lymphocytes in the chicken bursa of Fabricius undergo apoptosis by low doses of ionizing radiation [12]. However, it is still uncertain about the time course and dose-response for the induction of apoptosis in the bursa of irradiated chickens. Furthermore, there is little information on ultrastructural aspects of apoptotic cells in the bursa of irradiated chickens. Investigation of apoptosis induced in the bursa of irradiated birds would provide useful information on radiation-induced apoptosis of immature B lymphocytes, since our knowledge of radiation-induced

apoptosis of immature B lymphocytes, particularly *in vivo*, is much less than that of thymocytes.

In the present study, newly hatched chickens were subjected to whole-body X-irradiation, and the bursa was examined by light and electron microscopy to show the morphology of apoptotic cells, and to evaluate the time course and dose-response for the induction of apoptosis.

MATERIALS AND METHODS

Chickens and X-irradiation: Newly hatched male White Leghorn chickens were purchased from a local hatchery, and given whole-body X-irradiation within 1 day after hatching. The chickens, kept in a cardboard box, were irradiated at a dose rate of 0.43 Gy/min with a X-ray generator (Hitachi MS-30-5B) operating at 230 kV and 9 mA with a 1 mm Al + 0.25 mm Cu filter. Unirradiated chickens were retained in a cardboard box for a period of time equal to that required for delivering a given dose of X-irradiation, and served as controls.

Light microscopy: At 2, 4, 6, 12, 24 and 48 hr after irradiation, chickens were sacrificed by decapitation and the bursa was fixed with Bouin's solution. The organs were removed from at least two animals at each time point. The fixed tissues were dehydrated with a graded series of ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The fraction of pyknotic cells in total cells constituting the bursal follicle was determined by scoring pyknotic cells in a $75 \times 75 \mu\text{m}^2$ area of the bursal follicle. The determination was carried out on 20 randomly selected bursal follicles for each animal.

Electron microscopy: For transmission electron microscopy (TEM), the bursal tissues were obtained from

chickens sacrificed at 2 and 6 hr after X-irradiation of 8 Gy. The tissue specimens were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH7.5) for 24 hr at 4°C, and postfixed with 1% osmium tetroxide for 1 hr in the same buffer. The fixed tissues were dehydrated through a graded series of ethanol, embedded in Quetol 812, ultrathin-sectioned, stained with uranyl acetate and lead citrate, and then examined with a JEOL JEM-100S transmission electron microscope. For scanning electron microscopy (SEM), the bursal tissue was obtained from a chicken sacrificed at 4 hr after X-irradiation of 8 Gy. The tissue specimen was fixed in a mixture of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH7.4) for 24 hr at 4°C, and postfixed in 1% osmium tetroxide for 2 hr at 4°C. The fixed specimens were conductive-stained with 2% tannic acid and 2% osmium tetroxide for 4 hr each, dehydrated through a graded series of ethanol, and critical-point dried in liquid carbon dioxide. After sputter-coating, the specimens were examined with a JEOL JSM-T300 scanning electron microscope.

Analysis of DNA fragmentation by agarose gel electrophoresis: The bursa was dissected from a chicken sacrificed at 6 hr after irradiation. The bursal follicles were released into phosphate buffered saline by scraping the bursal folds with a scalpel blade. The bursal cell suspension was prepared by homogenizing the released follicles with a Dounce homogenizer followed by filtration through cotton wool. The bursal cells were pelleted by centrifugation, and low molecular weight DNA was extracted from the pelleted cells (1×10^8) essentially as described by Herrmann *et al.* [5], except that Triton X-100 was used instead of Nonidet P-40. The extracted DNA was separated by electrophoresis through 1.5% agarose gel, stained with ethidium bromide and then photographed under UV transillumination.

RESULTS

The bursa of Fabricius, a dorsal diverticulum of the cloacal proctodeum, contains numerous lymphoid follicles consisting mainly of developing immature B lymphocytes. Individual lymphoid follicles are known to consist of cortical and medullary regions in juvenile chickens. Nevertheless, as the cortical regions appear to start their growth from the last embryonic days [9, 14], these regions were still unclear in newly hatched chickens (Fig. 1a and b). A representative example of radiation-induced histological changes of the bursal lymphoid follicles is shown in Fig. 1c and d, where the bursa was observed at 6 hr after whole-body X-irradiation at 8 Gy. The lymphoid follicles became smaller and contained much more pyknotic cells than those of unirradiated chickens.

To assess the time course for induction of the pyknosis by X-irradiation, the percentage of pyknotic cells in the bursal follicle was determined at each time after whole-body X-irradiation at 8 Gy (Fig. 2). Although some pyknotic cells were observed in unirradiated chickens (about 4% of total constituent cells in the bursal follicle), X-

irradiation markedly increased the number of pyknotic cells. The percentage of the pyknotic cells increased sharply at 2 hr, reached a maximum (about 68% of total constituent cells in the bursal follicle) at 6 hr postirradiation, and then decreased to the control level at 24 hr postirradiation (Fig. 2). When the percentage of the pyknotic cells was examined at 6 hr after whole-body X-irradiation with doses ranging from 1 to 15 Gy, the evident induction of pyknotic cells was observed at 1 Gy, and the percentage of pyknotic cells increased with increments of the X-ray dose, reaching over 90% at 15 Gy (Fig. 3).

To demonstrate unequivocally that the observed pyknotic cells represent apoptotic cells, the bursa of irradiated chickens was examined by both TEM and SEM. TEM observation revealed many shrunken cells whose nuclei contained highly condensed chromatin and occasionally broke up into several fragments in the bursa of irradiated chickens (Fig. 4a). Despite the evident changes in the chromatin organization, the mitochondria appeared to be morphologically intact (Fig. 4b). Many cells with apoptotic morphology appeared to be engulfed by neighboring phagocytic cells. Furthermore, SEM observation revealed many roundish bodies in the bursa of the irradiated chicken (Fig. 5). These bodies not only varied in size, but also were considerably smaller than lymphocytes, indicating that these bodies represent apoptotic bodies. The morphological changes shown above were in good agreement with representative morphological characteristics of apoptosis such as cell shrinkage, chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies.

To further evaluate the induction of apoptosis by a biochemical criterion, low molecular weight DNA was extracted from the bursa of chickens at 6 hr after irradiation and separated by agarose gel electrophoresis. In unirradiated chickens, DNA bands corresponding to multiples of the nucleosomal size of 180–200 base pairs were very weak in intensity, substantiating the low incidence of apoptotic cells in the bursa of the unirradiated chickens (Fig. 6, lane c). In contrast, DNA extracted from the bursa of the irradiated chickens exhibited an intense DNA ladder pattern, which appears as a result of internucleosomal enzymatic cleavages of DNA and is a biochemical hallmark of apoptosis (Fig. 6).

DISCUSSION

The increase of pyknotic cells has long been known as a typical radiation-induced histological change in lymphoid tissues and organs, including the avian bursa of Fabricius [6, 11, 17]. The present study confirmed the marked increase of pyknotic cells in the bursa of newly hatched chickens after whole-body X-irradiation and clarified the ultrastructural characteristics of the pyknotic cells. The pyknotic bursal cells displayed typical apoptotic morphology such as cell shrinkage, chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies. In addition, most pyknotic cells appeared to be engulfed by

Fig. 1. The bursa of Fabricius of newly hatched chickens. a and b: unirradiated. c and d: 6 hr after whole-body X-irradiation at 8 Gy. (a, c) $\times 80$, (b, d) $\times 800$, HE stain.

neighboring phagocytic cells. Since macrophages are known to be present in the bursal follicle [1, 4], the phagocytic cells observed by TEM probably represent resident macrophages. It is known that phagocytosis of apoptotic cells before they lyse is also a typical feature of apoptosis in many tissues [2, 8, 20]. Thus, the present TEM and SEM observations showed explicitly that X-irradiation induced morphologically typical apoptosis in the chicken bursa. Moreover, the appearance of a DNA ladder on the agarose gel proved the induction of apoptosis in the bursa of irradiated chickens from a biochemical criterion.

Fig. 2. Time course of induction of pyknotic cells in the chicken bursa of Fabricius after whole-body X-irradiation of 8 Gy. Cont: unirradiated chickens. Data are shown as mean $\pm$ SD of 20 bursal follicles.

Fig. 3. Dose-response of induction of pyknotic cells in the chicken bursa of Fabricius at 6 hr after whole-body X-irradiation. Cont: unirradiated chickens. Data are shown as mean $\pm$ SD of 20 bursal follicles.

Conventional electron microscopy, such as that used here, is usually unable to identify the type of cells with the apoptotic morphology. However, it seems likely that the majority of the apoptotic cells observed in the bursal follicle are immature B lymphocytes, since nonlymphoid cells located in lymphoid tissues are known to be much more radioresistant than lymphoid cells and to be little affected by the doses of X-irradiation used in this study [6, 11].

As stated above, the pyknotic cells observed by light microscopy correspond to apoptotic cells, and therefore, the percentage of the pyknotic cells presented in the result is considered to be nearly, if not completely, equivalent to the percentage of the apoptotic cells. The pyknotic (apoptotic) cells evidently increased at 2 hr and reached a maximum at 6 hr after irradiation. The appearance of pyknotic cells within a few hours after irradiation is a common feature in most lymphoid tissues and organs, including the bursa of Fabricius [6, 11, 12, 17]. The present study showed that the percentage of the pyknotic (apoptotic) cells began to decrease after 6 hr and dropped to the control level by 24 hr. This decrease probably reflects the removal of the pyknotic (apoptotic) cells by resident macrophages, as suggested by the present TEM observation showing that most apoptotic cells were engulfed by phagocytic cells. The rapid and nearly complete removal of the apoptotic cells suggests that the chicken bursa possesses effective cellular systems by which apoptotic cells are recognized and removed rapidly.

The percentage of the pyknotic (apoptotic) cells showed a clear increase in the chicken bursa by X-irradiation at 1 Gy, and reached over 90% at 15 Gy. This dose-response indicates that most of the chicken bursal cells *in vivo* are very sensitive to X-irradiation with regard to induction of apoptosis. A large proportion (about 60%) of murine bone marrow immature B lymphocytes have been reported to

undergo apoptosis when cultured *in vitro* for 8 hr following gamma-irradiation at 5 Gy [3]. The present study shows that approximately 70% of bursal cells undergo apoptosis by 6 hr after irradiation at 8 Gy. Thus, immature B lymphocytes of chickens and mice seem to be equally sensitive to ionizing radiation *in vivo* and *in vitro* with regard to induction of apoptosis. It remains unknown at present whether radiation-induced apoptosis in immature B lymphocytes shares a common pathway in chickens and mice. Further studies are in progress to clarify this point.

The present study proved that chicken bursal cells readily undergo apoptosis when exposed to X-irradiation *in vivo*. We consider that the bursal cells serve as a useful model to evaluate the mechanism by which ionizing radiation induces apoptosis in immature B lymphocytes. Some viruses including infectious bursal disease virus have been reported to induce apoptosis in chicken lymphoid cells [7, 15, 16]. Although it remains unknown whether radiation and virus induce apoptosis by an identical mechanism, knowledge on radiation-induced apoptosis of the bursal cells may help to clarify the mechanism by which such viruses induce apoptosis.

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Fig. 4. Transmission electron micrographs of the bursa of Fabricius in newly hatched chickens. a: 2 hr after whole-body X-irradiation at 8 Gy. Arrows show cells whose nuclei broke up into two or three fragments. Bar=5 μ m. b: 6 hr after whole-body X-irradiation at 8 Gy. Intact mitochondria are still visible in the apoptotic cell indicated by the arrow. The arrow head shows the apoptotic cell engulfed and partially digested by a phagocytic cell. Bar=2 μ m.

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Fig. 5. Scanning electron micrographs of the bursa of Fabricius in newly hatched chickens. a: unirradiated. Bar=5 μ m. b: 4 hr after whole-body X-irradiation at 8 Gy. Many small roundish bodies (arrows) can be seen. Bar=5 μ m.

Fig. 6. Agarose gel electrophoresis of low molecular weight DNA extracted from the bursa of Fabricius. Newly hatched chickens were exposed to whole-body X-irradiation at 4 or 8 Gy, and the DNA was extracted from the bursa at 6 hr postirradiation. DNA from the bursa of unirradiated chickens is shown in lane C. Lane M shows DNA size markers (λ /HindIII digest) with positions indicated in base pairs.

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