Quantitative Detection of Apoptotic Thymocytes in Low-dose X-irradiated Mice by an Anti-single-stranded DNA Antibody

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The quantitative detection of apoptotic cells in low frequency in the thymus of mice irradiated with X-rays using an anti-single-stranded DNA antibody was explored. The antibody against single-stranded DNA (anti-ssDNA) was obtained with rabbits hyperimmunized with complexes of alkaline-denatured calf thymus DNA. AKR female mice were irradiated with 10 to 100 cGy or 4 Gy X-rays; thereafter, thymus sections were prepared at various times after irradiation. The detection and counting of apoptotic cells in the section were performed after histochemical staining using an anti-ssDNA antibody. The results demonstrate that, although sensitive and quantitative detection of apoptotic cells in irradiated thymus using the anti-ssDNA antibody is possible, the sensitivity is lower compared to that of in situ endlabeling methods, such as TUNEL or ISEL. The antibodies could also be used for rat thymus and spleen. In addition, an increase in positively stained cells by both methods was detected as early as 6 min after the irradiation of mice.

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INTRODUCTION

Apoptosis is essential for the appropriate development and function of multicellular organisms\(^1\), the malfunction of which, therefore, results in the initiation and progression of a number of human diseases\(^2\). The intense effort currently being devoted to studying of apoptosis is expanding knowledge of its process, the applications of which to cancer therapy are now being extensively discussed\(^3\). Furthermore, based on the cytological and biochemical characteristics of apoptotic cells, an increasing number of methods has been developed to detect and quantitate apoptotic cells in tissues.

A conventional and histological Haematoxylin and Eosin (HE) staining method is still commonly used\(^4\). The chromatin condensation characteristic of apoptosis is evident in cells as “pyknosis,” i.e., very dense staining of chromatin by haematoxylin. A growing number of flow-cytometry techniques have been developed for the analysis of cells undergoing apoptosis, despite its limitation, such as requiring a single-cell suspension\(^5\). In addition, several enzymatic techniques are currently used to detect DNA fragmentation in apoptosis at the cellular level. The most utilized one is the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end-labeling (TUNEL) method\(^6\). A similar method using DNA polymerase (here abbreviated as ISEL) is also extensively applied to paraffin-embedded tissue samples\(^7\). The single-stranded ends of fragmented DNA in apoptotic cells can be visualized by the incorporation of labeled nucleotides into the nuclei by these methods. Hence, the methods are essentially based on the single-stranded DNA in apoptotic cells.

We succeeded to produce an antibody against single-stranded DNA (anti-ssDNA)\(^8\), which was obtained with rabbits hyperimmunized with complexes of alkaline-denatured calf thymus DNA and methylated bovine serum albumin. The antibody was proved to be useful for detecting apoptotic cells in several tissues, including muscle, colorectal cancer, and hippocampus\(^9\text{–}11\). It could also detect cyclophosphamide-induced apoptotic cells in mouse thymic epithelial\(^12\). The applicability of the antibody for the detection and quantification of radiation-induced apoptosis in thymus has, however, not yet been examined.

In the preceding study we compared and evaluated the four methods mentioned above (HE, flow cytometry, TUNEL and ISEL) for identifying thymocyte apoptosis induced by low-dose X-irradiation\(^13,14\). We concluded that the in situ end-labeling method, especially ISEL, is the most adequate of the tested methods for low-frequency apoptosis, emphasizing those aspects important for the quantification of apoptosis in mouse thymus after X-irradiation with doses as low as cGy levels. In the present experiment, we extended such aspects as a comparative study to the anti-ssDNA antibody method. We measured the anti-ssDNA antibody’s ability to detect and quantitate apoptosis in thymus of mice irradiated with low-dose X-rays compared with ISEL and TUNEL. We further carried out a detailed time-course study on the appearance of anti-ssDNA antibody positive cells immediately after the irradiation in order to explore the possibility to utilize the antibody to detect ssDNA induced directly by X-rays prior to apoptotic DNA fragmentation.
MATERIALS AND METHODS

Animals
AKR/Jsea female mice (8 to 10 weeks old) were used throughout the experiment (both strains from Seac Yoshitomi, Ltd. Japan). The Wistar strain rats were also used for a species specificity examination of the anti-ssDNA antibodies. All of the animals were maintained on a light schedule from 8:00 to 20:00 and were given food and water ad libitum following the guidelines of the animal experiments of Toho University School of Medicine.

Preparation of an anti-single-stranded DNA antibody
An anti-ssDNA antibody was prepared as described elsewhere. Briefly, alkaline-denatured calf thymus DNA as immunogen and methylated bovine serum albumin produced an IgG antibody through 7-times injection to rabbits. The purified antibody was stored in 0.02% sodium azide.

X-ray irradiation and thymus section preparation
Mice were irradiated with X-rays as previously described with doses ranging from 10 to 100 cGy or 4 Gy using a Hitachi Medico MBR-1505 R2 X-ray machine operated at 150 kVp, 5 mA. Non-irradiated control animals were sham-irradiated in a Lucite chamber for the same duration as the irradiation. Thymus was removed at designated times immediately after irradiation up to 1 h or up to 6 h, and more than four mice were used in each experimental points. Tissues were fixed in 4% buffered formaldehyde, and then embedded in paraffin, according to the conventional histological procedures. Sections of 3–4 µm thickness were adhered to glass slides pretreated with 0.01% poly-L lysine (Sigma).

Detection and quantification of apoptotic cells
Sections adjacent to each other in the same paraffin section-ribbon were used for the preparations for a comparison. For example, the section used for ISEL was always adjacent to that used for the anti-ssDNA antibody method in an experiment in which the two methods were compared. Thus, a quantitative comparison of the methods was possible using the “same” tissue samples, but at “different” locations of 2–3 µm within the tissue. The procedures for ISEL and TUNEL were as previously described. Briefly, after deparaffinization, rehydration and a proteinase K treatment, the end labeling was performed using DNA polymerase or TdT for ISEL or TUNEL, respectively.

Procedures for Immunohistochemical staining using anti-ss-DNA
Immunohistochemical staining was carried out using an avidin-biotin-peroxidase system after deparaffinization and dehydration of paraffin-embedded preparations. In brief, after fixation, the preparations were sequentially exposed to normal goat serum (10%) for 30 min, IgG specific to single-stranded DNA (dilution 1:200) for 16 h, and biotinylated anti rabbit IgG for 10 min. The cells were then incubated for 5 min in an avidin-biotin-peroxidase solution. The
peroxidase activity was visualized by incubation with 3-3'-diaminobenzidine (DAB) tetrahydrochloride and 0.05% H₂O₂ in 0.1 M Tris-HCl (pH 7.6), followed by counter staining with 0.25% methyl green.

**Apoptotic cell count**

Positively stained nuclei or bodies in preparations were counted under a microscope at a magnification of 1000. More than one labeled apoptotic body lying in the same “halo” was judged as those have originated from one cell and was recorded as one count. Normal cells having normal nuclei (non-apoptotic cells) were counted at the same time. The percentage of apoptotic cells was calculated based on a count of more than 1000 cells.

**RESULTS**

**Identification of apoptosis in X-irradiated mouse thymi by immunohistochemical staining using an anti-ssDNA antibody**

DAB-positive cells could be clearly observed in anti-ssDNA antibody-treated sections of thymus in 4 Gy-irradiated mice (Fig. 1). Compared to sections prepared from non-irradiated control mice (Fig. 1a) and from mice immediately after the 4 Gy irradiation (Fig. 1b), the brown-colored positive cells increased remarkably in number in the section from the mice 4-h after irradiation (Fig. 1c). These results strongly suggest that the DAB-positive cells represent apoptotic cells induced by X-irradiation.

The results were confirmed by experiments in which double staining with TUNEL and the anti-ssDNA antibody of the same section was performed (Fig. 2). In this double-staining experiment, a new fuchsine dye was used for the anti-ssDNA antibody stain in order to differentiate red-colored, anti-ssDNA antibody-positive cells from brown-colored TUNEL-positive cells. Thus, double-positive cells can be easily recognized by their mixed, dark red color. The results show that the DAB-positive cells in ss-DNA treated section can indeed react to TUNEL, indicating that the positive cells correspond exactly to the apoptotic cells proved by TUNEL. Figure 2 further demonstrates that TUNEL can stain several DAB-negative cells in addition to the positive ones; namely, a part of the TUNEL-positive cells can be defined as apoptotic cells by the anti-ssDNA methods. In other words, the sensitivity of the anti-ssDNA labeling to detect apoptosis appeared to be lower compared with the in situ end labeling method, such as TUNEL and ISEL.

**Difference in the stain pattern between TUNEL and the anti-ssDNA antibody method**

In addition to the difference in sensitivity, the anti-ssDNA antibody stain method is distinct from TUNEL regarding pattern of staining. As shown in Fig. 3a, deep-brown colored dots are densely, but randomly, localized within a cell stained by an anti-ssDNA antibody. In contrast, the TUNEL-stained cell has a broad, diffusely but relatively uniformly stained area covering the whole nucleus, or occasionally extending to cytoplasmic region (Fig. 3b).
DETECTION OF APOPTOSIS BY ANTI ssDNA ANTIBODY

Fig. 1. Immunohistochemical detection of apoptosis in mouse thymus irradiated with 4 Gy X-rays. Thymus sections were prepared from non-irradiated control mice (Fig. 1a), from mice immediately (Fig. 1b) or 4 h (Fig. 1c) after the irradiation, and stained with anti-ssDNA antibody, as described in the text. Brown-colored positive cells can be clearly seen against the counter-stained background green-colored cells, and the positive cells are remarkably increased in number 4 h after the irradiation. The bars equal 100 µm.

Fig. 2. Thymus section stained simultaneously with TUNEL and with the anti-ssDNA antibody method. The thymus sections were prepared from mice 4 h after 4 Gy X-ray irradiation, and stained with TUNEL followed by the anti-ssDNA antibody staining with new fuchsine dye having red color. The arrow indicates the double-positive cells. The arrow head, a TUNEL-positive, but anti-ssDNA antibody-negative cell. The bar equals 10 µm.

Staining preparations from rat thymus and spleen with the antibody

Species specificity of the antibody is known to limit its applicability. The antibody used here could recognize both a DNA ladder and oligonucleosomes prepared from rat liver nuclei with endogenous endonuclease. The results shown in Fig. 4 confirm that the antibody is applicable to rat tissues. The figure shows sections of thymus (Fig. 4a) and of spleen (Fig. 4b) removed 4 h after 4 Gy irradiation from rat and stained with the anti-ssDNA antibody. The clustered brown-colored cells are distinctly visible from the surrounding green-colored cells in both figures.

Apoptosis-detecting sensitivity of the anti-ssDNA antibody technique

The percentages of apoptotic cells in thymus sections, as judged by ISEL or anti-ssDNA methods 4 h after irradiation of mice, are plotted against X-ray doses ranging from 10 to 70 cGy in Fig. 5. The lower sensitivity of the anti-ssDNA is clear. At any tested doses, less than...
about one-seventh ISEL positive cells were counted as apoptotic cells by the anti-ssDNA methods. However, both percentages increased with increasing radiation dose almost in parallel, suggesting similar responsiveness of both methods to an incremental increase in radiation.

Figure 6 shows the results of a time-course experiment in which changes in the ISEL- or anti-ssDNA-positive cell number were examined from 1 to 6 h after 50 cGy X-ray-irradiation. The number of ISEL-positive cells increased sharply from 2% at 2 h to more than 7% level at the peak of 4 h, then abruptly decreased to 2.5% at 6 h. The anti-ssDNA positive cell number, always about one-half of the ISEL-cells, exhibited change almost parallel to the ISEL positive cells’ change to 6 h after irradiation, except for the peak point at 4 h. The positive cell peak at 4 h is not as sharp as that of ISEL.

Appearance of TUNEL- as well as anti-ssDNA antibody-positive cells before 1 h after 4 Gy irradiation

In normal cells, single-strand breaks (SSBs) arise spontaneously during normal metabolism from a direct attack by reactive oxygen species. The exposure to 1 Gy of ionizing radiation gives rise to 100 direct SSBs per cell regardless of apoptosis induction \(^{15}\). It is therefore of interest to examine the possibility of the anti-ssDNA antibody labeling technique to detect
Fig. 5. Comparison of the two detection methods, ISEL and anti-ssDNA antibody stains, for apoptotic cells in X-ray-irradiated mouse thymus. To compare the detecting sensitivity of the methods, simultaneous assay by ISEL (open square with broken line) and anti-ssDNA antibody staining (open circle with solid line) was performed using the same thymus prepared from AKR mouse 4 h after irradiation with various radiation doses (10–70 cGy) shown on the abscissa. The percentage of apoptotic cells obtained by each method was plotted against the radiation dose. The vertical bars represent the ranges of ± SD. The differences in the percentages between the methods are statistically significant (p < 0.05).

Fig. 6. Changes in ISEL- or anti-ssDNA-positive cell number from 1 to 6 h after 50 cGy X-ray-irradiation. The percentage of the positive cells to ISEL (open square with broken line) or to anti-ssDNA antibody (open circle with solid line) was plotted against time after 50 cGy irradiation shown on the abscissa. The vertical bars represent the ranges of ± SD.

SSBs generally appearing before the onset of apoptosis in irradiated mouse thymus. We thus explored changes in the positive cell number within 1 h after 4 Gy irradiation, since we had previously found that no rise of the apoptotic cells occurred beyond the control levels at 1 h after irradiation16). Along with the anti-ssDNA labeling, we used TUNEL, expecting differen-
tional detection between apoptosis-“independent” (anti-ssDNA antibody positive) and -“dependent” (TUNEL positive) SSBs.

The time-course changes in the number of cells positive to both methods ran entirely in parallel, against our expectation, as shown in Fig. 7. The percentages of TUNEL positive cells rose steeply from the normal level of 1.5% to 2.6% at 6 min (statistically significant), but then gradually fell to 2% at 30 min, and again increased slowly to 2.5% at 1 h after irradiation (insignificant). Although entirely parallel changes were observed for the ssDNA positive cells, the percentages of the positive cells were much less than that of the TUNEL-cells (about one-fourth). It is clear from this that differential staining of SSBs directly induced by radiation from apoptotic SSBs is impossible for the ssDNA staining. The sensitivity of the method to detect SSBs, whether or not they are generated by apoptosis, is much lower than that of TUNEL.

Fig. 7. Changes in TUNEL- or anti-ssDNA-positive cells in mouse thymus immediately after 4 Gy X-ray-irradiation. The percentage of the positive cells to TUNEL (open square with broken line) or to anti-ssDNA antibody (open circle with solid line) was plotted against time up to 1 h after 4 Gy irradiation. The vertical bars represent the ranges of ± SD.

DISCUSSION

The use of an anti-ssDNA antibody to detect apoptotic cells in normal as well as tumor tissues has already been reported. Frankfurt et al developed a novel immunohistochemical procedure for the staining of apoptotic cells using a monoclonal antibody (MAb) to single-stranded DNA. This MAb stained all cells with the morphology typical of apoptosis in etoposide-treated HL-60, MOLT-4, and R9 cell cultures. Further, they successfully applied this MAb for the detection and precise quantitation of apoptotic cells in solid tumors, such as breast carcinoma.

Our present data support and extend these earlier observations by suggesting that the
anti-ssDNA antibody is useful to detect and quantitate apoptotic cells in thymus of mice irradiated with X-rays. An antibody- and TUNEL-double staining experiment using the same specimen (Fig. 2) and a comparative quantification study (Figs. 5–7) clearly demonstrates that the response of the antibody staining method to a subtle change in apoptosis frequency is sufficient, although the sensitivity of the anti-ssDNA antibody staining to detect apoptotic cells is low compared to that of TUNEL and ISEL.

The difference in the sensitivity to detect apoptotic cells between the antibody method examined here and TUNEL or ISEL could possibly be, at least partially, interpreted in terms of relatively the high, non-specific background frequency of TUNEL- or ISEL-positive cells in thymus.

The difference in stain pattern between antibody staining and TUNEL (Fig. 3) suggests that the fine molecular structure of single-stranded DNA reacting to the antibody or to TdT in TUNEL is different from each other. The antibody used here consists of several antibodies, which recognize hexadeoxynucleotides with various base sequences. The available data are, however, not sufficient to discuss this matter further.

The findings obtained by the 1 h-time-course experiment performed immediately after irradiation (Fig. 7) are inconsistent with our current knowledge concerning the timing of onset of DNA fragmentation. It is generally known that, in irradiated thymus, the late execution phase leading to chromatin DNA breakages by endonuclease(s) begins about 1 h or later following irradiation. Therefore, we had expected that no change in the TUNEL-positive cell number occurred within 1 h after irradiation, whereas the antibody-positive cells could change due to SSBs induced directly by radiation. The results suggest, however, that no differential detection among both methods is possible, and that apoptotic (positive to both staining methods) cells appeared as early as 6 min after irradiation, and showed an exactly similar pattern for both methods.

An explanation for these results is that SSBs induced by radiation disappeared rapidly by an efficient repair process after irradiation, and then there was no cells responding to the antibody staining at the time of the experiment. An alternative interpretation is also possible by assuming that the antibody used could not react against the radiation-induced SSBs; instead, it responds exclusively to ssDNA associated with apoptosis. Indeed, the antibody could recognize both a DNA ladder and oligonucleosomes prepared from rat liver nuclei with endogenous endonuclease. Thus, these results claim the induction of positively stained cells by both methods as early as 6 min after irradiation. The implications and mechanisms of these phenomena remain to be investigated.

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