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Original

Detection of Apoptotic Cells in a Dexamethasone-Induced Thymic Apoptosis Model in Rats Using A Modified Warthin-Starry Silver Impregnation Method to Prevent Fading

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Abstract: A modified Warthin-Starry (mWS) method was developed to stain apoptotic cells in tissue sections. Essentially the classical WS method is applied except for a minor change to prevent fading. In order to clarify the relationship between mWS-positive cells and apoptosis, apoptotic thymic tissues were examined on the rats treated with dexamethasone. The results clearly showed the distribution of mWS-positive cells to coincide well with that for apoptotic cells observed by terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end labeling (TUNEL) method. An additional characteristic is that nuclei of mitotic thymocytes, with highly condensed chromatin, are always stained. These observations suggest that the mWS method might be useful and convenient to detect condensed chromatin during apoptosis. (J Toxicol Pathol 2001; **14**: 225–230)

Key words: silver staining, TUNEL, apoptosis, thymocyte

Introduction

Apoptosis has been a target of intensive studies in cell biology and cancer research for it has profound implications for many aspects of cellular processes. In contrast to necrosis, apoptosis is a cell suicide that requires energy supply from mitochondria and is morphologically characterized by marked chromatin aggregation, cell shrinkage, loss of cell-to-cell contact, and quick removal by macrophages[1–3]. Important in this respect is the ability to detect apoptotic events in early stages or even prior to its onset. A difficulty is that cells go through many different routes depending on their differentiation type before arriving at the common feature of strong chromatin condensation. Of a variety of methods so far developed[4–11] for the identification of apoptosis in tissue sections, the most widely is perhaps the terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end labeling (TUNEL) method[12]. However, it is becoming more and more clear in recent years that nicking is not directly related to apoptosis because it is neither necessary nor sufficient for the process. Therefore, it is desirable to detect factors, which induce strong chromatin condensation that may lead to apoptosis.

During a histopathological study of Helicobacter gastritis, we incidentally noticed that some nuclei at the borders of erosions and/or ulcers were stained by the Warthin-Starry's (WS), silver-impregnation method[13, 14]. The stained cells were not necrotic but possibly apoptotic by reason of their pyknotic nuclear appearances on H&E stained sections. The present investigation was undertaken to clarify the relationship between WS positive staining and apoptosis. Dexamethasone (DM), a synthesized glucocorticoid, has been reported to induce rat thymic apoptosis *in vivo*, with apoptotic thymocytes clearly increasing 8–24 hours after treatment at a dose of 1 mg/kg[15]. In the present study, the WS method was modified to prevent fading and applied to DM-induced apoptotic thymic tissue in rats.

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Materials and Methods

Animals and experimental design

A total of 27 male 7-week-old Slc:SD rats were obtained from SLC Japan Inc. (Shizuoka, Japan) and divided into 2 groups: 24 animals were intraperitoneally injected

with DM solution (Orgadrone®, Sankyo Co. Ltd, Tokyo, Japan; dissolved in saline) at a dose of 0.5 mg/kg body weight, and the others were used as controls. Three animals each were sacrificed at 1, 2, 4, 8, and 24 hours, and 3, 7, and 14 days after the treatment. The controls were sacrificed at 24 hours after saline injection. All animals were killed under ether anesthesia. The thymus was removed from each rat, and fixed in 10% phosphate buffered formalin solution for 24 hours.

Histopathological examination

Tissues were routinely processed for embedding in paraffin, sectioned and stained with hematoxylin and eosin (H & E).

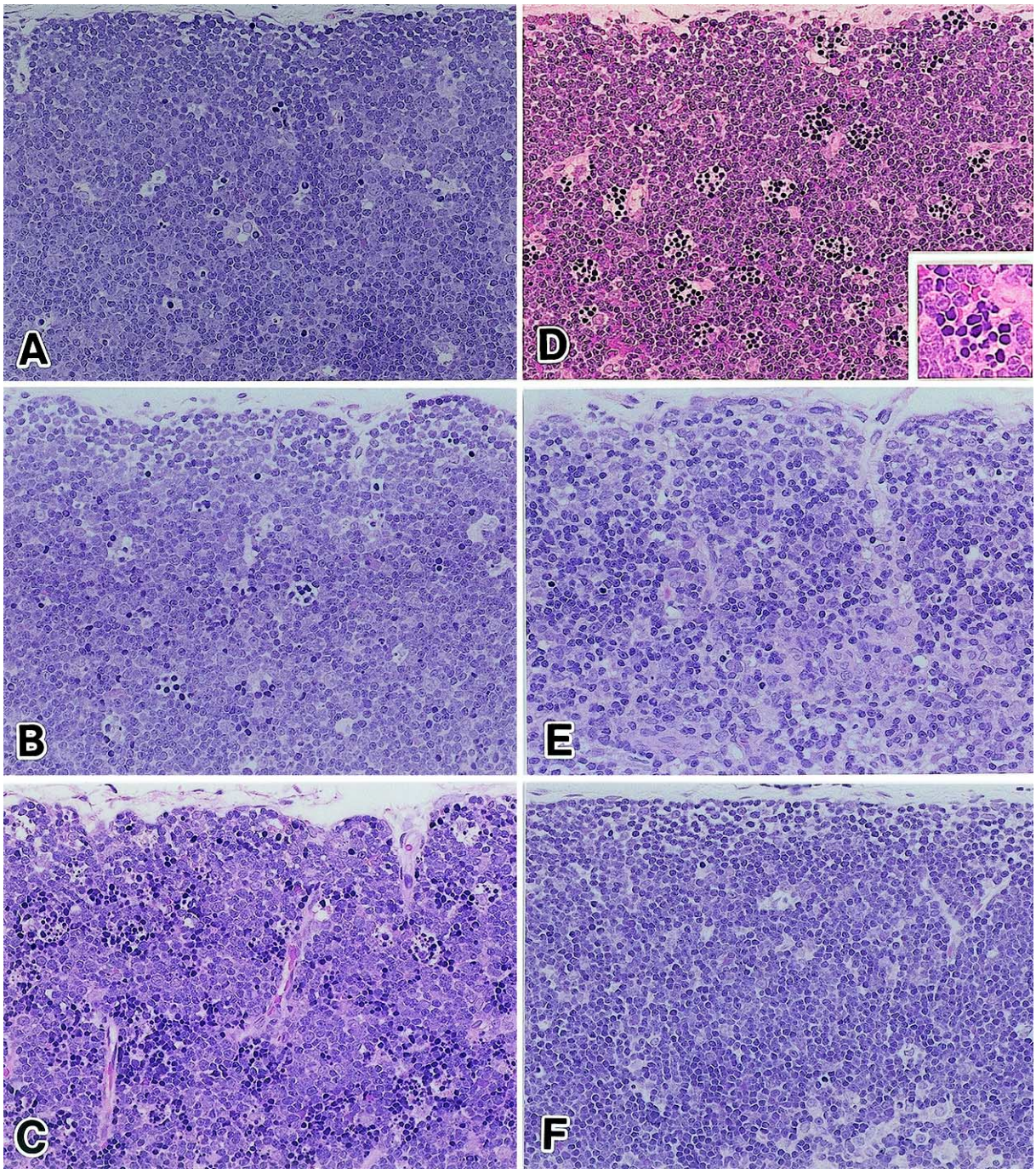

Fig. 1. Sequential changes of thymus at 1 hours (A), 2 hours (B), 8 hours (C), 24 hours (D), 3 days (E), and 14 days (F) after DM treatment. The number of pyknotic thymocytes in the cortex was increased from 2 hours after the injection, reaching a maximum 8–24 hours after the injection and the decreasing. The thymus had completely recovered from the influence of the injection at day 14. H & E stain. Magnification: × 260. Inset in D: A higher magnification of pyknotic nuclei of thymocytes. Magnification: × 530.

Our modified WS (mWS) staining was performed on paraffin sections from all the animals. The staining method is as follows. Deparaffinized sections were washed several times with distilled water, treated with 1% silver nitrate solution in acidified water (adjusted with 1% citric acid to a pH of 4.4) for 30 min at 37°C. A mixture of 0.15% hydroquinone, 5% gelatin, 2% silver nitrate at 8:6:15 was used for development. Sections were reacted for 3 to 5 min at room temperature and washed several times with distilled water. In addition to the classical WS method, they were fixed by using an acid hardening fixer for photographic film and paper (FUJIFIX; Fuji Photo Film Co. Ltd., Tokyo, Japan) to prevent fading. Then, they were counterstained with nuclear fast red, dehydrated through a graded ethanol series, cleared with xylene and mounted with Entellan New® (Merck Japan Ltd, Tokyo, Japan).

To detect nick ends in nuclei associated with apoptosis, tissue sections from all rats were also stained by the TUNEL method using a manufactured kit (TACS™ *in situ* apoptosis detection kit, Trevigen, Inc., MD, USA). Sections were developed with diaminobenzidine hydrogen peroxidase or TACS blue label substrates. Counterstaining was accomplished with methyl green or nuclear fast red.

Serial mirror sections were made from an animal sacrificed at 8 hours after DM treatment. One section was stained by the mWS method, and the other by the TUNEL method.

For electron microscopic examination, small pieces of thymus from one animal 8 hours after DM injection were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) and post-fixed with 2% osmium tetroxide in the same buffer, for 2 hours each, immediately after sampling. Samples were embedded in Epok 812 (Ouken Shoji, Co., Ltd., Tokyo, Japan). Ultrathin sections were obtained with an ULTRACUT-E microtome (Reichert-Jung, Nussloch, Germany), double-stained with uranyl acetate and lead citrate, then observed under a transmission electron-microscope (JEM-1200EX, JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

Results

In the sections stained with H&E, small foci of pyknotic nuclei and nuclear fragments were observed in a sparse distribution in the cortex 2 hours after injection of DM. With the lapse of time, the number of foci increased together with the number of pyknotic nuclei within each focus. The numbers reached a maximum 8–24 hours after injection and then decreased (Figs. 1A-F). Electron-microscopic observation of apoptotic nuclei revealed a characteristically condensed and homogenous chromatin distribution (Fig. 2).

TUNEL-positive cells also appeared in the cortex of thymus 2 hours after injection of DM. The spatial distribution roughly coincided with the regions where nuclear fragments and pyknotic nuclei existed. The distribution of TUNEL-positive cells essentially overlapped the pyknotic thymocytes. The number reached a peak 4–24 hours after the injection and returned to the normal by the 7th day (Figs. 3A-C).

Nuclei of thymocytes showed two staining patterns with the mWS method; one intense diffuse staining of condensed nuclei and the other staining of the nuclear periphery. The diffuse staining pattern was most characteristic of apoptotic cells. The time course in change in numbers of affected cells was well correlated with the results of the TUNEL method (Figs. 3D-F). Furthermore, serial mirror sections demonstrated a good coincidence of mWS-positive cells and TUNEL-positive cells (Figs. 4A, B). An additional characteristic was that mitotic thymocytes, which have highly condensed chromatin, were also consistently stained.

Discussion

Various methods, which have been developed to detect apoptotic cells in tissue sections, can be divided into two major categories. The first focus on cellular substances which appear in association with apoptosis, before or after onset. Le^y antigen defined by its reactivity with a mononuclear antibody BM-1/JIMRO is recognized as an apoptosis-related cytoplasmic protein[10]. Other targets for detection include apoptosis-related protein or mRNA[5], cathepsin D[8] and tissue transglutaminase[9]. The second category relies on demonstration of DNA damage, e.g. DNA denaturation or strand breaks, that occur most probably as a result of extreme condensation of chromatin. Antibodies against single-stranded DNA are reported to be good markers of drug-induced apoptosis and programmed cell death during embryogenesis[4, 11, 16], and several enzymatic techniques have recently been developed[12, 17]. The most widely used among them is the TUNEL method which detects nick ends in tissue sections and this has been

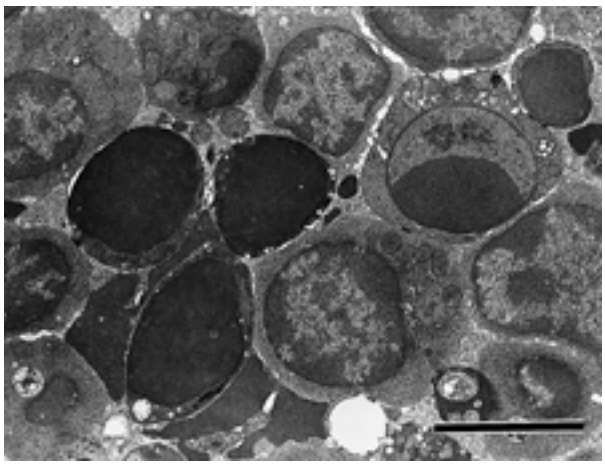

Fig. 2. Thymus from a rat 8 hours after DM treatment. Apoptotic thymocytes show a characteristically condensed and homogenous chromatin distribution. Electron microscopy. Bar = 4 μm.

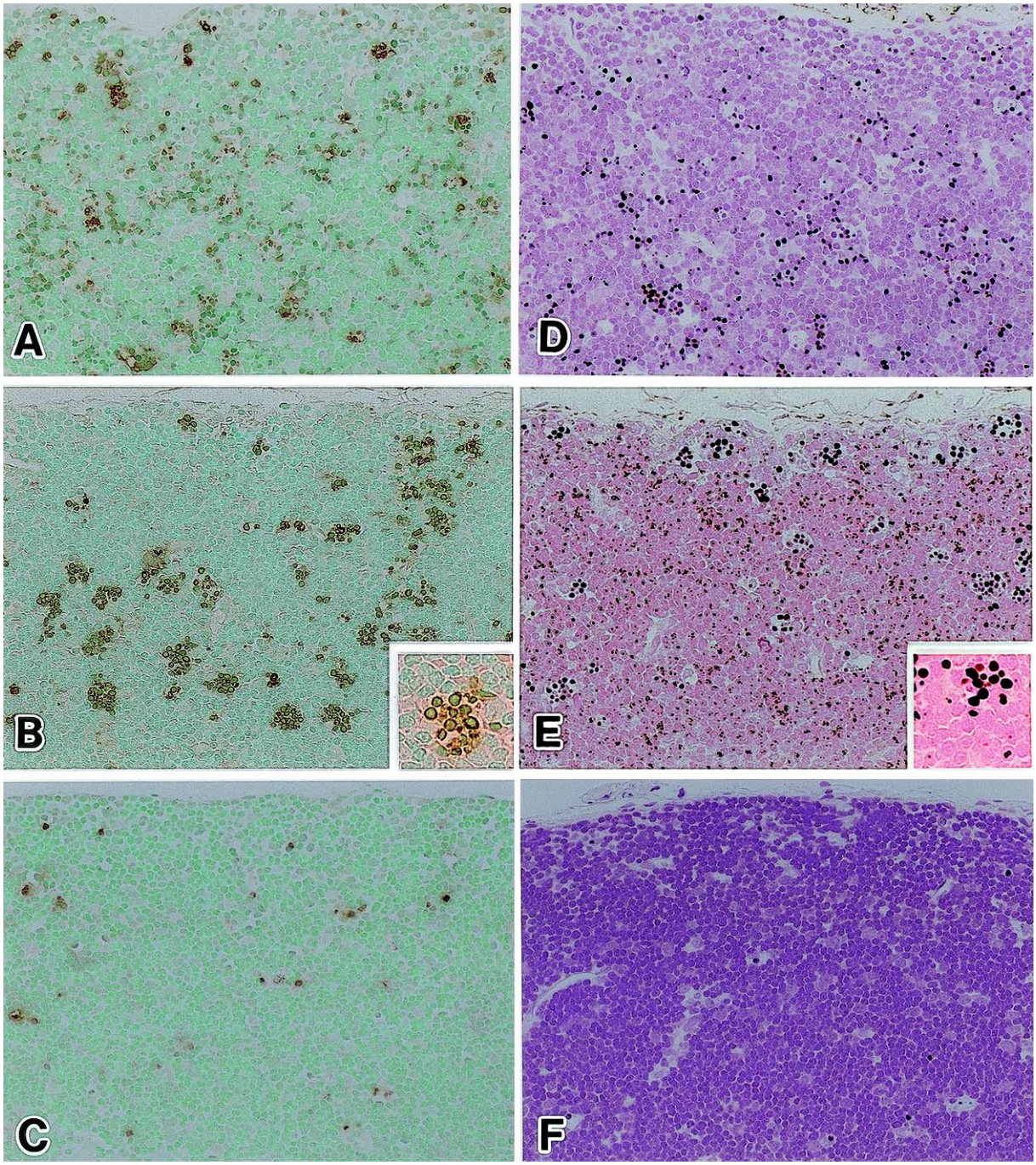

Fig. 3. Microscopic features of thymus tissue at 2 hours (A and D), 24 hours (B and E) and 14 days (C and F) after DM treatment. Positive cells with both TUNEL and mWS methods essentially overlap with pyknotic thymocytes. A, B and C: TUNEL method. D, E and F: mWS stain. Magnification: × 260. Insets in B and E: higher magnifications of TUNEL and mWS positive thymocytes, respectively. Magnification: × 530.

extensively applied to analyze tumor progression[16, 18, 19]. However, to the authors' knowledge, no report has addressed the significance of silver impregnation methods for the detection of apoptotic cells. We examined several silver-impregnation protocols, the reticulin sliver impregnation, Grimelius' method, Fontana-Masson's method, and silver staining of nucleolar organizer regions (AgNORs), but it showed that mWS method was the only one that can be used to stain rat's apoptotic thymocytes with reproducible results (unpublished data).

In the present study, mWS staining demonstrated condensed thymic nuclei and allowed clear detection of sequential changes with chemical induced apoptosis. Although the mitotic thymocytes containing condensed

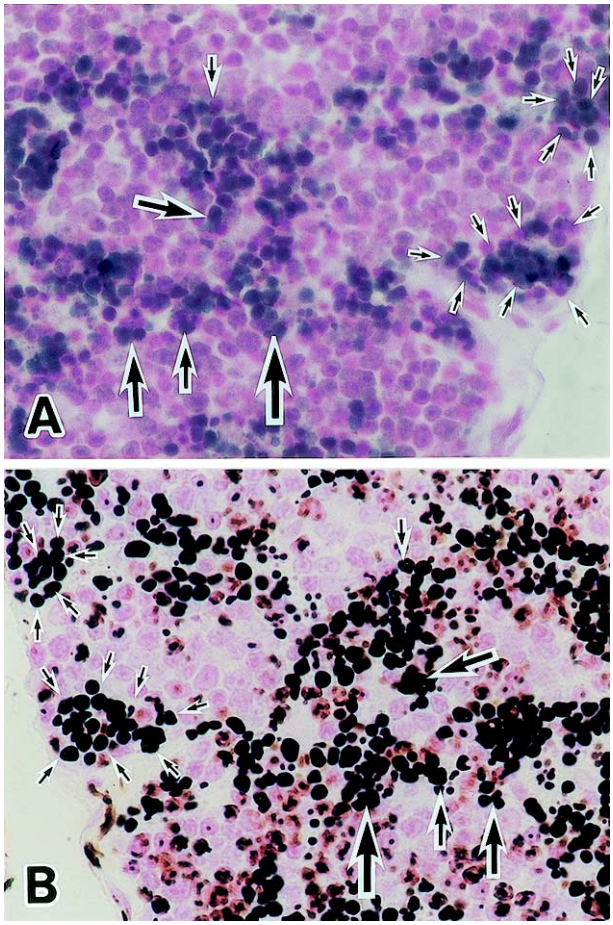

Fig. 4. Serial mirror sections of thymus from a rat 8 hours after DM treatment stained with TUNEL (A) and mWS (B) methods. Arrows on each figure indicate the matched foci of positive cells on each section. Note the good coincidence of mWS-positive and TUNEL-positive cells. Magnification: × 530.

chromatin were also stained, comparison with H&E stained sections allowed clear differentiation of cell types. Apoptosis is a phenomenon that was originally defined on the basis of its morphological characteristics, especially marked condensation of chromatin[1]. From this viewpoint, the results of mWS staining might be the best index to apoptotic cell death. Besides, we tried staining a few rat liver sections which had spontaneous focal hepatocellular necrosis with mWS method, with the results that there were no positive reaction for necrotic nuclei (unpublished data). Therefore, mWS staining could be considered as a specific method to detect apoptosis, and the staining procedure is simple and less time-consuming and costly than the TUNEL method.

The mechanism why mWS method detects apoptosis is unknown. However, it is of interest for us that mWS staining reacts not only with intense diffuse condensation of chromatin but also with nuclear periphery which is considered to be a heterochromatin region characterized by focal condensation of chromatin and with mitotic nuclei. These results might probably suggest that mWS staining detects some nuclear substance related with the processes of chromatin condensation and/or heteropyknosis. Further studies of this method are necessary.

In conclusion, mWS staining might be a useful and convenient method to pathologically diagnose apoptotic cells.

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