FULL PAPER *Anatomy*

Immunoelectron Microscopic Observation of Chicken Glucagon-Like Peptide (GLP)-1-Containing Cells in Tissues Derived from Thin Section, Paraffin Block and Conventional Method

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ABSTRACT. The purpose of the present study was to investigate the possibility of immunoelectron microscopic observation of endocrine cells in paraffin-embedded tissues. The procedure, which involves reprocessing from sliced tissues and immunohistochemical staining by colloidal-gold immunolabeling of paraffin sections from paraffin blocks, was able to reveal the fine characteristics of secretory granules containing glucagon-like peptide-1. Morphometric analyses of the secretory granules showed no significant differences between the reprocessing procedure and a conventional post-embedding procedure, which was performed as a control. The reprocessing procedure has some advantages besides providing information on secretory granules containing the amino acid peptide. For example, the same cell can be observed under both a light microscope and the electron microscope. In addition, the high-electron densities of silver-enhanced gold particles are easily recognized, and the boundary between the profile of the granules and the immunogold labeling is clearly shown at the electron microscopic level. Furthermore, the procedure, which is inexpensive and does not require special devices, can effectively use precious samples that are already paraffin-embedded and unable to be obtained twice, such as the case for endangered animals and rare pathological tissues. To the best of our knowledge, the present study is the first to report the advantages of the reprocessing method for sliced paraffin sections of gut endocrine cells.

KEY WORDS: glucagon-like peptide-1, immunocytochemistry, paraffin-embedded tissue, reprocessing method, transmission electron microscope.

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There can be no question that immunohistochemical methods have made tremendous contributions to scientific progress. Immunohistochemistry (IHC), which detects cellular antigens, provides the best visualization of histological structures and the exact light microscopic localizations of immunolabeled probes [2]. The first choice of tissue preparation for light microscopic observation is mostly fixation with formalin, an aqueous solution of formaldehyde or paraformaldehyde solution and paraffin embedding. Formaldehyde/paraformaldehyde at various concentrations and in combination with other chemicals and buffers is the most commonly used fixative and preserves the general structures of cells and extracellular components by reacting with amino groups [18]. Since formaldehyde/paraformaldehyde does not cause significant protein denaturation, the proteins maintain their abilities to react with specific antibodies. This property is important for IHC. Formaldehyde/paraformaldehyde-fixed and paraffin-embedded samples make permanent preservation possible, and large quantities of samples, not only as paraffin blocks but also as thin slices from paraffin blocks and immunohistochemically stained tissue sections, are stored at many laboratories.

Conversely, immunoelectron microscopy is a useful method for observing detailed and satisfactory morphological information. Therefore, pre- and post-embedding techniques for immunoelectron microscopy are widely used [2]. These methods require advanced and special preparation techniques at the time of specimen procurement [27]. After observation by IHC at light microscopic level, researchers sometimes note that the samples should have been treated for immunoelectron microscopic analysis. However, reprocurement of samples, such as those from endangered animals and rare pathological tissues, can be difficult. Specimens obtained from reprocessing of paraffin-embedded tissues are generally thought to be unsuitable for electron microscopic observation. There are several techniques available for electron microscopic obser-

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vation of paraffin-embedded tissues, and these methods are used for surgical pathology material [27]. Lighezan *et al*. [8] reported that organelles, such as nuclei, desmosomes, sarcomeres, intermediate filaments, tonofilaments and electron-dense granules, can be recognized by transmission electron microscopic observation, while organelles, such as mitochondria, polyribosomes, lipids and glycogen, exhibited various degrees of degradation in reprocessed paraffin-embedded tissues. No reports about the possibility of observing hormones and the influence of reprocessing on the ultrastructure of gut endocrine cells have been documented to the best of our knowledge. Although Mar *et al*. [9] described immunocytochemical observation of rat growth hormone-containing cells between light microscopic images and electron microscopic images of the same cell, they used one plastic-embedded sample, but not a paraffin-embedded sample.

We selected and observed chicken glucagon-like peptide (GLP)-1 as an experimental hormone in the present study. It is well documented that GLP-1 is a 30-amino acid peptide that is released from intestinal L-cells after meal ingestion [3, 12, 15]. This meal-induced gut hormone has many important physiological actions, especially the potentiation of glucose-induced insulin secretion, thereby lowering the plasma glucose levels in mammals [3, 5, 11, 12]. Our previous study clarified the ultrastructural characterization of chicken GLP-1 in secretory granules in L-cells using post-embedding techniques [12]. In the present study, we investigated the possibility for immunoelectron microscopic observation of this endocrine cell in paraffin-embedded tissues. Three kinds of ultra-thin sections prepared by different techniques were compared at the transmission electron microscopic level: 1) a reprocessing sample from paraffin sections that had been immunohistochemically stained by the linked streptavidin-biotin method, which had undergone complete light microscopic observation and been coverslipped with a mounting agent 1 year or more before; 2) a reprocessing sample from sliced paraffin sections from paraffin blocks immunohistochemically stained by the colloidal-gold immunolabeling method; and 3) a post-embedding sample as a control experiment for the first 2 pre-embedding samples for immunoelectron microscopy.

MATERIALS AND METHODS

Experimental birds: Seven-day-old White Leghorn male chickens were used in this study. The birds were fed a commercial balanced diet and water *ad libitum* and maintained under controlled light conditions (12-hr light/12-hr dark). At the end of the experimental period, the birds were euthanized, and their tissues were used as described below. The birds were treated in accordance with the Guideline for Regulation of Animal Experimentation (1997) of the Faculty of Agriculture, Shinshu University.

Tissue samples: The experimental methods were divided into three kinds as follows: a pre-embedding technique for immunoelectron microscopy reprocessed from paraffin sections stained by the linked streptavidin-biotin method (PrLSB) that had been coverslipped with Entellan® (Merck, Darmstadt, Germany) and completed light microscopic observation 1 year or more before; a pre-embedding technique for immunoelectron microscopy reprocessed from paraffin sections that had been newly sliced at a thickness of 5 μm from previously embedded paraffin blocks and immunohistochemically stained by colloidal-gold immunolabeling (PrCG); and a post-embedding technique for immunoelectron microscopy from sections newly sampled and stained by colloidal-gold immunolabeling (PoCG). Paraffin sections in both PrLSB and PrCG were mounted on MAS-GP coated glass microscope slides (Matsunami Glass Industries Ltd., Osaka, Japan).

The birds (n=4) used for PrLSB and PrCG were perfused with physiological salt solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.6). The distal ileum was immediately dissected out after the perfusion and cut into small blocks with razor blades. The tissue blocks were cut open along the mesenterium, stretched on a cork plate, immersed in the same perfusate at 4°C overnight, washed several times with phosphate buffer and embedded in paraffin wax according to standard procedures.

The birds (n=4) used for PoCG were perfused with 4% paraformaldehyde and 0.01% glutaraldehyde in phosphate-buffered saline (PBS). The distal ileum was sliced under a dissecting microscope into 1 × 1 × 2-mm blocks, immersed in the same perfusate at 4°C overnight, dehydrated through a graded series of ethanol and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan).

Immunoelectron microscopy: The processing for each electron microscopic technique is described below.

PrLSB. The linked streptavidin-biotin staining was visualized with 0.05% 3,3′-diaminobenzidine (DAB) as described in our previous paper [10]. Briefly, the following antisera were used: rabbit antiserum against synthetic human GLP-1 (amino acids 1–19) (1:2,000 dilution; GA1176; Affiniti Research Products, Devon, U.K.), which does not cross-react with other proglucagon-derived peptides [20]; and biotinylated goat anti-rabbit IgG serum (diluted 1:300; AP132B; Millipore, Billerica, MA, U.S.A.). Sections on glass microscope slides were soaked in xylene for 12 hr to remove the coverslips and then rehydrated through a decreasing graded series of ethanol for 5 min each. After washing with PBS, the sections were examined under a light microscope, and the intestinal mucosa containing GLP-1-positive cells was dissected into 1 × 1-mm pieces. The sections were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min, postfixed in 1% osmium tetroxide (OsO_4) for 15 min, dehydrated through a graded series of ethanol and embedded in Quetol 812. After polymerization of the resin, the sections were detached from the glass slides by warming. Ultra-thin sections (80 nm) were obtained using an ultramicrotome (Super Nova; Reichert-Jung, Vienna, Austria), mounted on 200-mesh nickel grids (Nisshin EM), stained with 10% (v/v) TI Blue (Nisshin EM) and 2% (w/v) lead citrate and observed under a transmission electron microscope (JEM-1400; JEOL, Tokyo, Japan).

PrCG. Paraffin sections were newly cut at 5 μm thickness from blocks embedded 1 year before. After routine dewaxing and rehydration, the sections were pretreated with 2.5% normal donkey serum (IHR-8135; Immunobioscience, Mukilteo, WA, U.S.A.) for 20 min and incubated

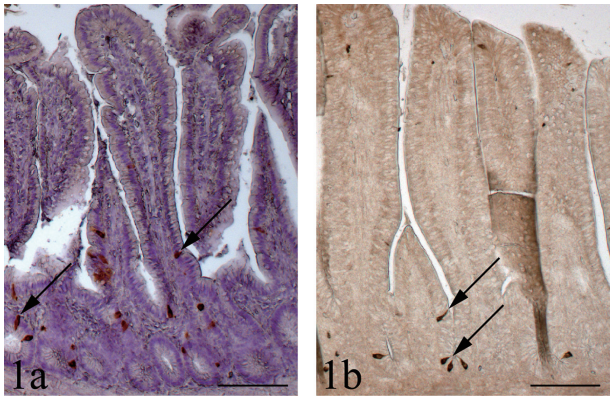

Fig. 1. Immunohistochemical findings of GLP-1-positive cells in the chicken ileum. (a) A section stained with the PrLSB procedure and counterstained with Mayer's hematoxylin. (b) A section stained with the PrCG procedure. The immunoreactive cells are distributed in the crypts and lower part of the villous epithelium (arrows). Bars=200 μm.

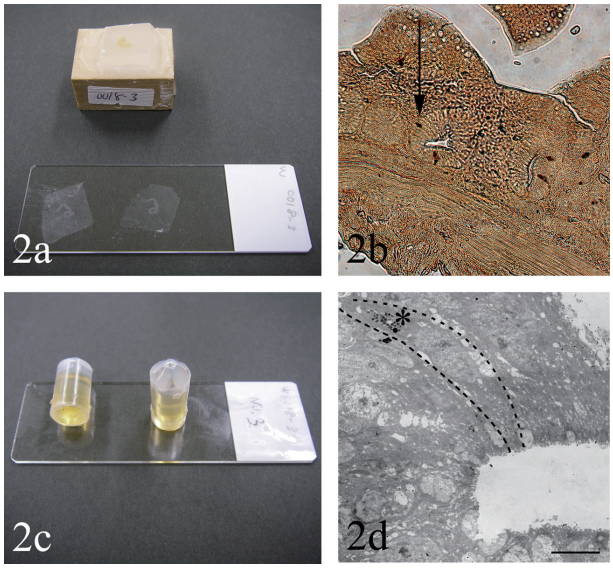

Fig. 2. Summary of the PrCG procedure from a paraffin block to electron microscopic observation. (a) Distal ileum embedded in paraffin and paraffin sections mounted on a glass slide. (b) A section stained with the PrCG procedure examined under a light microscope without a coverslip. The arrow indicates an immunoreactive cell observed under the electron microscope in panel d. (c) Dissected sections including immunoreactive cells embedded in Quetol 812. The left block was detached from the glass slide by warming. (d) Electron micrograph of an immunoreactive cell. The cell surrounded by a dashed line is the same cell indicated by the arrow in panel b. Secretory granules accumulate in the basal cytoplasm of L-cells (asterisk). Bar=5 μm.

with the above-described rabbit antiserum against synthetic GLP-1 (1:2,000 dilution) for 12 hr. After three washes with PBS, the sections were incubated with colloidal gold (6-nm diameter)-labeled donkey anti-rabbit IgG serum (1:100 dilution; 711–195-152; Jackson ImmunoResearch, West Grove, PA, U.S.A.) for 3 hr. The sections were then immersed in 2.5% glutaraldehyde for 15 min and serially processed for the silver-enhanced colloidal gold method [25] by immersion in 0.1 M acetic acid buffer (pH 7.0) for 10 min to yield detailed electron-immunoreactive structures and enhancement using a silver enhancement kit (HQ Silver; Nanoprobes, Yaphank, NY, U.S.A.) in a dark room for 8 min. After thorough washing with 0.1 M phosphate buffer, dissection, postfixation with OsO_4, embedding in Quetol 812 and electron microscopic observation were performed as described for PrLSB.

PoCG. Ultra-thin sections from blocks embedded in Quetol 812 were obtained as described above. The ultra-thin sections were pretreated with 1% (w/v) sodium periodate solution for semi-deresination to readily allow reactions between the antibody and antigen and incubated with 1% normal donkey serum for 15 min. After three washes with PBS, a mouse monoclonal primary antibody against synthetic human GLP-1 (amino acids 7–36) (1:8,000 dilution; A6104.1; Immundiagnostik, Bensheim, Germany) was applied to the sections for 24 hr. After three washes with PBS, the sections were treated with colloidal gold (12-nm diameter)-labeled donkey anti-mouse IgG serum (1:200 dilution; 715-205-150; Jackson ImmunoResearch) for 2 hr, stained with 10% (v/v) TI Blue and 2% (w/v) lead citrate and observed under a transmission electron microscope. The specificity of the primary antibody used in this study has been documented by the manufacturer and in previous reports [4, 12, 21].

Morphometry of GLP-1 granules: Morphometric analyses were carried out to characterize the secretory granules of L-cells. L-cells that contained a nucleus and more than 10 granules were selected for morphometric analyses. A total of 471 secretory granules from 15 cells in PrLSB, 195 secretory granules from 10 cells in PrCG and 207 granules from 12 cells in PoCG located at the basal or perinuclear region of the cytoplasm were randomly selected. The shortest and longest diameters of the secretory granules were measured using an image analyzer (KS 400; Carl Zeiss, Oberkochen, Germany). Differences between the mean diameters of granules from each method were tested by ANOVA and with a selected Tukey–Kramer test for multiple comparisons. The significance level was set at 0.01.

RESULTS

Immunoreactive cells for the GLP-1 antiserum, so-called L-cells, were observed in the full length of the distal ileum in PrLSB and PrCG under a light microscope. The cells were distributed in the epithelium of the crypt and the lower part of the intestinal villi and had comma- or flask-like shapes (Fig. 1a and 1b). Figure 2 shows results of the PrCG procedure from a paraffin block to electron microscopic observation.

At the lower magnification electron microscopic level, the GLP-1-containing cells were easily identified as precipitated dark products resulting from DAB in PrLSB and gold particle enhanced by silver reactions in PrCG (Fig. 3a and 3b). On the other hand, it was difficult to identify the granules labeled with the 12-nm-diameter gold particles under low magnification in PoCG. Although degradation of the nucleoplasm and cytoplasm was observed, secretory granules could

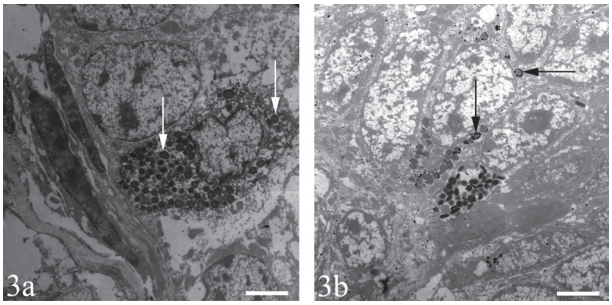

Fig. 3. Low-magnification electron micrographs of the chicken distal ileum stained with the PrLSB (a) and PrCG (b) procedures. GLP-1-positive cells are easily identified at low magnification by the dark DAB reaction products and gold particles enhanced by silver reactions (arrows). Degradation of the nucleoplasm and cytoplasm is observed in both procedures. An enterochromaffin cell is observed adjacent to the GLP-1-positive cell in panel (b). Bars=2 μm.

Table 1. Shortest and longest diameters of the secretory granules in the different procedures

Procedure	Shortest diameter (nm)	Longest diameter (nm)
PrLSB	311 ± 70*	378 ± 80*
PrCG	288 ± 58	355 ± 69
PoCG	291 ± 56	343 ± 66

Data are shown as the mean ± standard deviation. *P<0.01, significant difference from the other procedures.

be identified in PrLSB and PrCG (Fig. 3a and 3b). These cells contained round to oval secretory granules without a halo that mainly accumulated in the basal cytoplasm or perinuclear region of L-cells (Fig. 4a–4c). The electron densities of the secretory granules observed among the three different experimental methods were medium and almost the same. As shown in Fig. 4b, the immunopositive granules for GLP-1 had lower electron density than the secretory granules of adjacent enterochromaffin cells, which were widely distributed in the gastrointestinal tract and had polymorphous secretory granules.

Examination under higher magnification clarified the differences in the immunoreactivities among the three different experimental methods. In PrLSB, the DAB reaction products precipitated around secretory granules and within the cytoplasm (Fig. 4d). In PrCG, the gold particles enhanced by silver products mainly precipitated around secretory granules, and a few were located in the cytoplasm (Fig. 4e). In PoCG, the gold particles were mostly found on secretory granules, and very few of them were located in the cytoplasm (Fig. 4f). The profile of the secretory granules was clearly observed in PrCG and PoCG, but was difficult to identify in PrLSB (Fig. 4d–4f). Because the electron density of the DAB reaction product was similar to that of the secretory granules, the deposition of DAB reaction products around the whole profile of the granules tended to make the boundary between the granules and cytoplasm ambiguous. The electron density of the DAB reaction product was quite low compared with that of the gold particles (Fig. 4e and 4f).

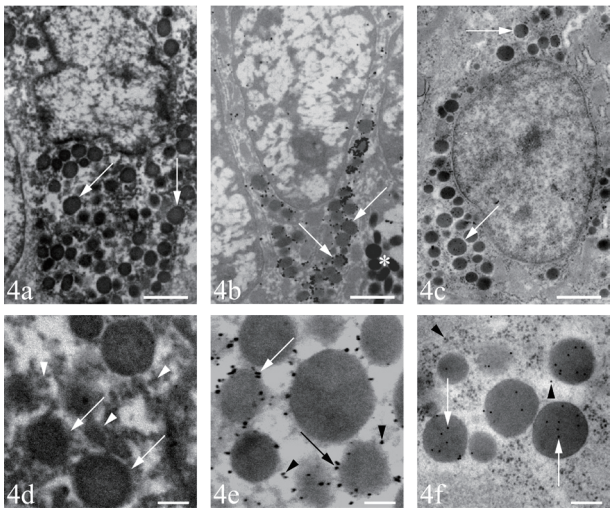

Fig. 4. Electron micrographs of GLP-1-positive cells in the chicken distal ileum stained with the PrLSB (a), PrCG (b) and PoCG (c) procedures. The cells contain round-to-oval secretory granules without a halo. The electron densities of the secretory granules observed with the three different experimental methods are medium and almost the same (arrows). The secretory granules of an enterochromaffin cell adjacent to the GLP-1-positive cell show polymorphous shapes and high-electron density (asterisk) (b). The post-embedding procedure shows fine preservation of the nucleoplasm and cytoplasm (c). Bars=1 μm. (d) High-magnification view of the secretory granules in panel (a). The DAB reaction products precipitate around the secretory granules (arrows) and in the cytoplasm (arrowheads). The profile of the granules is ambiguous. (e) High-magnification view of the secretory granules in panel (b). The particles of enhanced colloidal gold mainly precipitate around the secretory granules (arrows), and a few of them are located in the cytoplasm (arrowheads). The profile of the secretory granules is clearly observed. (f) High-magnification view of the secretory granules in panel (c). Almost all of the particles of colloidal gold are diffusely arranged on the secretory granules (arrows), and very few of them are located in the cytoplasm (arrowheads). The profile of the secretory granules is very clearly observed. Bars=200 nm.

The results of the morphometric analyses of the secretory granules are shown in Table 1. The longest and shortest diameters of the secretory granules in PrLSB were significantly longer than those in both PrCG and PoCG.

DISCUSSION

Immunoelectron microscopy is a useful method that provides detailed morphological and biological information. It is mainly classified into three methods, pre-embedding, post-embedding and non-embedding (ultra-thin frozen section) methods. These three methods each have advantages and disadvantages, and researchers are required to select the most effective method appropriate to the tissues under observation. Pre-embedding immunocytochemical staining has provided successful observations by light microscopy and electron microscopy in the same specimen [9, 16]. However, permeabilization treatments are sometimes necessary to obtain optimal labeling of intracellular antigenic sites, particularly with thick sections, such as vibratome sections [7, 9, 14]. The use of penetration agents, such as enzymes, or detergents, such as Triton X-100, often

leads to destruction or disorganization of more labile cytoplasmic structures and frequently gives rise to false-positive staining [9]. In contrast, post-embedding immunocytochemical staining of ultra-thin sections at the electron microscopic level produces high-resolution localization and good morphological preservation. Epoxy resin is one of the best media for this technique. However, epoxy resin blocks a number of antigenic epitopes through interactions with tissue components and forms a hydrophobic polymeric network that prevents the diffusion of water-soluble substances, such as antibodies [2, 6]. To expose antigenic sites to the immunoreagents, etching of epoxy resin ultra-thin tissue sections with reagents, such as periodic acid or hydrogen peroxide, is necessary [2, 12, 23]. Some researchers have taken advantage of hydrophilic embedding media, such as LR White/Gold embedding resin, to avoid this problem. In either case, post-embedding procedures tend to prevent antibody binding to a sufficient number of antigenic sites, which frequently gives rise to false-negative staining [9]. Indeed, the primary antibody used in PrLSB and PrCG did not react in PoCG in the present study, although the specificity of the primary antibody has been proven. A non-embedding approach, which uses ultra-thin frozen sections and colloidal gold, can overcome the penetration problem and also preserve the tissue antigenicity [9, 19]. This procedure has the advantages of not requiring penetration agents or chemical fixatives. However, the method requires specialized equipment and an acquired skill of sectioning [9].

Paraffin-embedded tissues have been used for immunohistochemical staining for a long time. The medium of paraffin allows tissues to be thinly sliced, typically in the range of 5–15 μm. The specimen is rehydrated through a decreasing graded series of ethanol, which facilitates the penetration of antibodies. In this study, we attempted to observe 5-μm sections that were cut from paraffin blocks and immunostained for observation under an electron microscope. It has been thought that ultrastructural examination of paraffin-embedded tissues is associated with disruption of membranes, loss of clarity and varying degrees of tissue degradation for reasons, such as series of hydration/dehydration processes. However, some studies have documented that electron microscopic observation produced the opposite result [8, 17]. Lighezan *et al*. [8] reported that the ultrastructural features of paraffin-embedded tissues that were reprocessed for electron microscopic observation were quite variable and also pointed out that the fixation, rather than the paraffin embedding, seemed to be most important step that affected the quality of the ultrastructural features. Moreover, they documented that optimal initial fixation in formalin at an appropriate concentration and pH allowed ultrastructural preservation and that ultrastructural examination of paraffin-embedded tissues could be optimized by avoiding paraffin blocks prepared from poorly fixed tissues and tissues adjacent to areas of necrosis. Some studies have clearly documented that organelles, such as nuclei, desmosomes, sarcomeres, intermediate filaments, tonofilaments and electron-dense granules, can be recognized from reprocessed paraffin-embedded tissues under electron microscopic observation, although mitochondria, polyribosomes, microtubules, lipids and glycogen can exhibit various degrees of degradation [8, 22, 24, 26]. The above-mentioned studies on reprocessed paraffin-embedded tissues were performed on newly cut sections from paraffin-embedded blocks, but not on already sliced paraffin sections attached to the glass. To our knowledge, a study about the possibility of observing hormone-containing endocrine cells reprocessed from paraffin-embedded tissues has not been documented. In the present study, we investigated the possibility not only of endocrine cells in the conventional paraffin section with pre-embedding immunocytochemical electron microscopic observation but also from newly sliced paraffin sections that were subjected to immunostaining. We observed secretory granules containing chicken GLP-1 using PrLSB and PrCG techniques.

At the electron microscopic level, the immunopositive secretory granules in PrLSB and PrCG could be recognized more easily than those in PoCG under low magnification, owing to the precipitated dark products resulting from DAB and gold particles enhanced by silver reactions. Incubations with antisera and other reagents were performed before the ultra-thin sectioning in PrLSB and PrCG. In addition, the incubations were performed after the ultra-thin sectioning in PoCG. Immunoreaction sites were identified around the secretory granules in PrLSB and PrCG and on the content of the granules in PoCG. Although the granules had the same shape among the three procedures, both the longest and shortest diameters of the secretory granules were significantly longer in PrLSB than in both PrCG and PoCG. The electron density of the DAB reaction product was similar to that of the secretory granules in PrLSB, which made the boundary of the granules ambiguous. Baskin *et al*. [1] suggested that osmium-DAB interactions resulted in background problems. Because OsO_4 is well known as a post-fixative solution, it was used in PrLSB and PrCG. We presumed that this use led to the result that the diameter of the measured granules tended to be longer in PrLSB.

Conversely, the gold particles enhanced by silver products mainly precipitated around secretory granules, and a few of them were located in the cytoplasm in PrCG. Since the electron density of the silver-enhanced gold particles was much higher than that of the granules, the boundary between the granules and the immunogold labeling was clearly recognized under both low and high magnifications. It was presumed that this clarity provided similar evaluation of the granule diameters between PrCG and PoCG. Because of the difficulty in obtaining sagittally sectioned electron micrographs of L-cells that contained many granules, the measured numbers of granules differed among the three groups. This study is the first to identify the ultrastructure of secretory granules containing an amino acid peptide (GLP-1) obtained from paraffin-embedded and sliced sections with similar evaluation results to PoCG, which is a commonly used method for post-embedding immunocytochemical studies. The reprocessing method from thinly sliced paraffin sections in the present study has limitations compared with reprocessing from paraffin blocks. The reprocessing method from paraffin blocks, in which paraffin-embedded tissues are dewaxed in xylene, hydrated, postfixed, dehydrated and embedded in epoxy resin, shows fine preservation of nuclei and many cytoplasmic organelles [8, 13, 22, 24, 26]. The nucleoplasm and cytoplasm had poor preservation in PrLSB and PrCG. PrLSB showed worse preservation and

more tissue degradation. We presumed that this resulted from removal of the coverslip and repeated hydration/dehydration procedures. However, PrCG provided satisfactory information for the secretory granules containing the amino acid peptide hormone. The fixative solution used in PoCG contained glutaraldehyde, which is commonly used for electron microscopic studies and preserves the fine structure of nuclei and many cytoplasmic organelles. We presumed that the fine preservation in PoCG was also caused by glutaraldehyde.

To the best of our knowledge, the present study is the first to report the advantages of the reprocessing method with sliced paraffin sections, which not only provides information on secretory granules containing an amino acid peptide, but is also inexpensive, does not require special devices and can effectively use precious samples that are already paraffin-embedded and unable to be obtained twice, such as the case for endangered animals and rare pathological tissues. In addition, this method may contribute to ethical ideas that have led to a required reduction in the number of laboratory animals used by researchers. In particular, PrCG, which is summarized in Fig. 2, is a useful procedure from the following viewpoints: 1) the thinly sliced and hydrated sections allow penetration of antibodies; 2) the gold enhanced by silver provides fine information of immunoreactive cells at the light microscopic level; and 3) the investigator can observe the same cell under a light microscope and the electron microscope.

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