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Detection and Evaluation of Proliferating Cell Nuclear Antigen (PCNA) in Rat Tissue by an Improved Immunohistochemical Procedure

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Abstract

Proliferating cell nuclear antigen (PCNA), an endogenous cell replication marker, was detected by an improved immunohistochemical procedure (super sensitive biotin-streptavidin), in formalin fixed, paraffin embedded tissue. Small intestine, liver, kidney, testis, and esophagus from rats were stained for the detection and evaluation of PCNA as a suitable marker for cell proliferation. Different patterns of staining that are believed to correlate with the individual phases of the cell cycle (G_1, S, G_2, M, G_0) were recognized. PCNA offers an alternative to established cell labeling techniques for investigating cell proliferation.

Key words: formalin fixation, paraffin processing, proliferating cell nuclear antigen, super sensitive biotin-streptavidin

Introduction

A variety of methods have been used to detect normal and transformed proliferating cells. Stathmokinetic agents (ie, colchicine, vinblastine), initially used for the detection of proliferating cells, function by arresting cells in metaphase (1,2). This method was replaced by the more reliable and accurate technique of autoradiography with tritiated thymidine as a labeling agent. Autoradiography is an effective means of determining the cell labeling index (labeled cells in S phase/labeled plus unlabeled cells). However, this method is expensive, laborious, and results are not rapidly obtained. A technologist trained in handling radioactive labeled isotopes is required to contain and decontaminate areas exposed to tritiated thymidine properly. Routine emulsion exposure is 1 to 4 weeks or longer (3). Strict quality control must be followed to obtain reliable, interpretable results. An alternative method for identifying S phase cells is 5-bromo-2'-deoxyuridine (BrdU).

BrdU, a thymidine analog, is incorporated into cells actively undergoing DNA synthesis and may be subsequently quantitated by flow cytometric or immunohistochemical procedures. Flow cytometry is limited by the requirement of cell suspensions; therefore, the exact tissue localization of specific S phase cells is not possible. However, if immunohistochemical techniques are used to identify the BrdU antigen, individual S phase cells are readily localized. Immunohistochemical detection of BrdU is less time consuming than autoradiography. Cell labeling indices can be calculated the same day of staining. Care is required when handling BrdU because it is classified as a potential carcinogen.

Although these methods provide accurate results, both tritiated thymidine and BrdU must be pre-administered to identify DNA replicating, S phase cells. For this reason, cell proliferation research is done primarily in experimental systems such as cell cultures and animal models. These reagents are administered via pulse dosing or by surgically implanted osmotic minipumps. Labeling indices depend upon the duration of exposure to the administered labeling compounds, (and, in the case of thymidine autoradiography, on the duration of photographic emulsion exposure). Pulse dosing must be administered in the correct time frame to identify S phase cells. Variables such as diurnal cell replication cycles can lead to inaccurate labeling interpretation (4). Osmotic minipumps help to alleviate these variables in that they can be implanted in experimental animal models for up to 14 days. A method to identify replicating cells that does not encompass elaborate technical preparation, safety hazards, and excessive costs and time had not been developed until the recent commercial availability of an antibody that recognizes the naturally occurring cell replication marker, proliferating cell nuclear antigen (PCNA).

PCNA is a 36,000 molecular weight auxiliary protein of DNA polymerase delta, an enzyme vital for DNA replication (5-7). Synthesis of this protein begins in the late G_1 phase and peaks during the S phase of the cell cycle (7-10). Studies have indicated PCNA to be a highly stable protein found in all tissues (10). Investigators measuring cell replication via immunohistochemistry (IHC) have identified PCNA in normal and transformed proliferating cells (10-11). However, the detection of replicating cells using PCNA by IHC has lacked sensitivity in paraffin embedded tissues. Using an improved IHC procedure on formalin fixed, paraffin embedded tissues, we were able to intensify PCNA staining, make more efficient use of the antibody, and reduce the primary incubation time without enzyme predigestion. Improved detection of PCNA in formalin fixed, paraffin embedded tissues will facilitate routine assessments of cell replication.

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Materials and Methods

Tissues

The small intestine, liver, kidney, testis, and esophagus of untreated, 8 week old, Sprague Dawley rats were fixed in 10% buffered formalin for 18–24 hr and transferred to 70% ethanol overnight. Tissues were processed in a Tissue-Tek VIP processor (Miles Scientific, Elkhart, IN). All temperatures for processing and embedding were kept under 60°C. Samples were paraffin embedded, sectioned at 6 μm, and mounted on poly-L-lysine coated slides. Sections were air dried overnight, deparaffinized in xylene, and hydrated through a graded series of ethyl alcohol to 1×Automation Buffer, pH 7.5 (Biomeda Corporation, Foster City, CA). The super sensitive biotin-streptavidin method for immunohistochemical localization was performed as follows:

Reagents

1. 1×Automation Buffer, pH 7.5 (Biomeda)
   10×Automation Buffer–100.0 ml
   Distilled water (D/W)–900.0 ml
2. 2N hydrochloric acid (Stable at room temperature)
   D/W–417.0 ml
   Concentrated hydrochloric acid–83.0 ml
3. 3% Hydrogen peroxide (Make fresh)
   30% Hydrogen peroxide–1.0 ml
   D/W–9.0 ml

Antibody Dilutions

1. Diluent
   Mix 1 gm bovine serum albumin (BSA) with 100 ml 1× Automation Buffer. Refrigerate.
2. Blocking serum: Normal goat serum (Vector Laboratories, Burlingame, CA)
   Normal goat serum–100.0 μl
   Diluent 1.9 ml
3. Primary antibody: Mouse monoclonal antibody to PCNA (19A2) (19A2; #660-4541) (Coulter Immunology, Hialeah, FL) (Make fresh)
   Dilution 1:400
   Mouse monoclonal antibody to PCNA–10.0 μl
   Diluent–3.9 ml
4. Link and label antibody/(StrAviGen™ Super Sensitive Prediluted Immunostaining Kit, Biogenex Laboratories, AP500-5M, San Ramon, CA)
   Link–Prediluted (biotinylated anti-immunoglobulins, specific for mouse)
   Label–Prediluted (streptavidin conjugated to horseradish peroxidase)
   These kits may be purchased concentrated or prediluted. If using the concentrated kits, dilute the link and label antibody with diluent to 1:10.

Staining Procedure

1. Tissue sections were placed in 2N HCl at 37°C for 20 min.
2. Slides were washed in 2 changes of 1×Automation Buffer 5 min each.
3. Endogenous peroxidase was quenched by placing slides in 3% hydrogen peroxide for 10 min.
4. Slides were washed in 2 changes of 1×Automation Buffer, 5 min each.
5. Slides were treated with normal goat serum (Biogenex) at a dilution of 1:20 for 20 min at room temperature (RT) to reduce the nonspecific binding of the linking antibody.
6. Blocking serum was drained from the slide and excess antibody wiped from around the tissue sections.
7. Sections were incubated with primary antibody, PCNA (19A2) at a dilution of 1:400 for 30 min at RT. Negative control slides from each of the same tissues were incubated with control antibody (normal nonimmunized mouse immunoglobulin in diluent) in place of the primary antibody for 30 min.
8. Slides were washed in 2 changes of 1×Automation Buffer, 5 min each.
9. Sections were incubated with link antibody (biotinylated anti-immunoglobulins, specific for mouse) (StrAviGen™ Super Sensitive Prediluted Immunostaining Kit) for 30 min at RT.
10. Slides were washed in 2 changes of 1×Automation Buffer, 5 min each.
11. Label antibody (streptavidin conjugated to horseradish peroxidase) (StrAviGen™ Super Sensitive Prediluted Immunostaining Kit) was applied to sections for 30 min at RT.
12. Slides were washed in 2 changes of 1×Automation Buffer, 5 min each.
13. Prior to chromogen staining, the sections were rinsed in D/W for 3 min. Aminoethylcarbizole (AEC) (AEC Peroxidase Chromogen Kit, Biomeda Corporation) was used as the chromogen. Slides were incubated 45 min at RT.
14. Slides were rinsed in D/W, counterstained in hematoxylin, blued in 1×Automation Buffer, wiped dry and mounted with Crystal/Mount (Biomeda).
16. The mounted sections were polymerized in an 80°C incubator for 15 min. After cooling, the slides were dipped in xylene and permanently mounted with Pro-Texx (American Scientific Products, McGaw Park, IL).

The blocking, primary and control antibodies were diluted with a diluent composed of 1% bovine serum albumin in 1× Automation Buffer.

Results

Positive staining of PCNA antigen-antibody complexes was observed in all tissues examined (Figures 1–6). The staining results were categorized based upon cellular distri-

Figure 1. Photomicrograph of rat small intestine stained for PCNA and counterstained with hematoxylin. Several crypt nuclei are bright red indicating cells in S phase. Other cells have a diffuse, speckled nuclear and cytoplasmic staining. A mitotic cell (arrow) has diffuse, speckled cytoplasmic staining. Original magnification × 500.

Figure 2. Photomicrograph of a rat kidney cortex stained for PCNA. Two tubular epithelial nuclei stain distinctly positive for PCNA. Original magnification × 500.

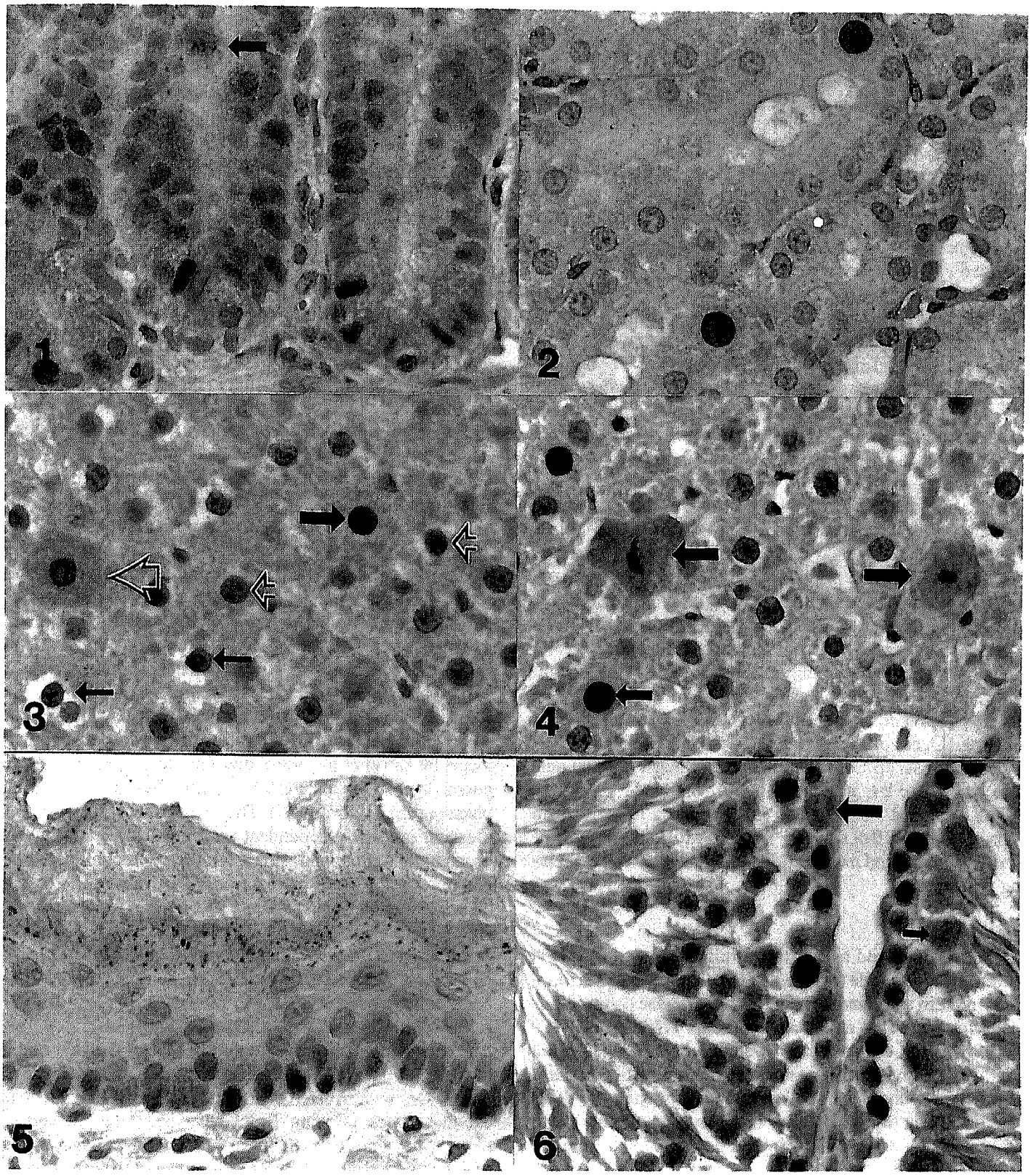

Figure 3. Photomicrograph of rat liver stained for PCNA and counterstained with hematoxylin. The dark red staining nucleus (large arrow) represents a cell in S phase. Unstained nuclei (small arrows) represent cells in G_0. Several nuclei that have a light reddish tint (small open arrows) are in late G_1. The hepatocyte with a condensed nucleus and speckled cytoplasmic staining (large open arrow) is in late G_2 just prior to going into mitosis. Original magnification × 500.

Figure 4. Photomicrograph of rat liver stained for PCNA showing two mitotic cells (large arrows) with granular cytoplasmic staining. At least one hepatocyte nucleus (small arrow) that is stained dark red is in S phase. Original magnification × 500.

Figure 5. Photomicrograph of rat esophagus stained for PCNA with a hematoxylin counterstain. Several basal cells in the stratified squamous mucosa have deep red nuclear staining characteristic of S phase of the cell cycle. Original magnification × 500.

Figure 6. Photomicrograph of rat testis stained for PCNA and counterstained with hematoxylin. Early germ cells along the basement membrane are stained deeply red, whereas several spermatocytes show less intense staining. Sertoli cells (large arrows) and some spermatocytes (small arrows) are negative for PCNA, indicating they are in G_0. Original magnification × 500.

bution and intensity of the reaction product. For positive staining, the categories of cellular distribution were: uniform nuclear staining; diffuse, speckled nuclear and cytoplasmic staining; diffuse, speckled cytoplasmic staining. Staining intensity was graded as 1+ (light pink), 2+ (red), or 3+ (deep red to black).

Staining was most prominent in the basal crypt cells of the small intestine. Positively stained cells had 2+ uniform nuclear staining or 2+ speckled nuclear and cytoplasmic staining (Figure 1). Cells with mitotic figures had 2+ diffuse, speckled cytoplasmic staining (Figure 1).

Distinct 2+ to 3+ staining was detected in some renal tubule nuclei (Figure 2), although an occasional renal tubule cell had minimal (1+) nuclear staining with absence of cytoplasmic staining. Infrequent mitotic cells had 2+ diffuse, speckled cytoplasmic staining. Minimal (1+) nuclear staining was evident in the glomerular mesangial cells. These staining features exemplify the documented low cell turnover rate in the kidney (12).

All positive PCNA staining patterns were observed in the liver. Kupffer cells exhibited 2+ nuclear staining, but staining of bile duct epithelium was infrequent. Hepatocytes had 1+ to 3+ uniform nuclear staining; 2+ diffuse, speckled nuclear and cytoplasmic staining; and 2+ diffuse, speckled cytoplasmic staining, especially evident in cells containing mitotic figures (Figures 3 and 4).

In the esophagus, positive staining was evident in many cells in the basal layer of the stratified squamous epithelium with 2+ to 3+ uniform staining of these nuclei (Figure 5). However, sporadic basal cell nuclei had 2+ diffuse, speckled staining. Occasional stratified epithelial cells located between the basal epithelium and the keratinized luminal surface had 1+ to 2+ nuclear staining.

The testis had 3+ positive PCNA staining in early germ cells with 1+ to 2+ staining in spermatocytes (Figure 6). Interstitial cells and most sertoli cells lacked a positive staining product.

Discussion

With an improved biotin-streptavidin amplified detection system, we were able to detect and evaluate PCNA, an auxiliary protein associated with DNA polymerase delta, in formalin fixed, paraffin embedded rat tissue. This improved detection system, commercially available in kits, is supplied with a link and label antibody. The link antibody, biotinylated anti-immunoglobulins, specific for mouse, allows the attachment of numerous biotin molecules without negatively affecting antibody binding affinity. The procedure for conjugation of the enzyme label to streptavidin has been optimized to permit maximum labeling of the streptavidin molecule with multiple enzyme molecules. Together the link and label antibody increases the amount of signal generated per antigen-antibody binding event relative to any of the standard indirect procedures, (ie, peroxidase anti-peroxidase, avidin-biotin complex, biotin-streptavidin). Previous attempts in our laboratories to amplify the PCNA antigen-antibody complex via the avidin-biotin complex method yielded inadequate, unacceptable staining. The improved biotin-streptavidin amplified detection system permitted increased staining intensity of the PCNA antigen, made more efficient use of the antibody, and reduced the primary incubation time without the need for enzyme predigestion. Other investigators have attempted to identify PCNA in formalin fixed tissue by standard indirect IHC techniques with variable success, possibly because of decreased staining as a function of prolonged exposure to fixative (personal observation) (13–16). The studies were done primarily in human tissue with fixatives other than formalin (13–15). The identification of the protein by IHC methods in formalin fixed, paraffin embedded tissue has not been successfully demonstrated until now.

Detection of PCNA by IHC techniques may yield more information about the cell cycle than previously speculated. Molecular studies indicate the synthesis of PCNA is initiated in the nucleus in late G_1 phase and continues during the S phase (10). The different staining patterns recognized in this study are believed to reflect individual phases of the cell cycle. Cells expressing no staining in the nucleus or cytoplasm are expected to be quiescent G_0 phase cells. Minimal (1+) nuclear staining would be consistent with G_1 phase cells since PCNA synthesis begins in the late stages of this phase. The distinct and intense 2+ and 3+ nuclear staining would then identify cells in S phase where PCNA production is greatest. In preparation for mitosis, the cell enters another gap phase. During this second gap phase the nuclear envelope begins to dissipate. In G_2 the DNA polymerase delta and its auxiliary protein, PCNA, would leak into the cytoplasm with dissolution of the nuclear envelope, allowing identification of G2 cells by a 2+ diffuse, speckled nuclear and cytoplasmic staining. In mitosis the nucleoplasm and cytoplasm coalesce with the loss of nuclear boundaries. This could account for the diffuse speckled cytoplasmic staining specifically observed in all actively mitotic cells. Experiments by Kurki et al (1986) lend further support to the proposed hypothesis of PCNA staining within the various phases of the cell cycle (10). Their results indicate PCNA expression is greatly dependent on the cell cycle. Using immunofluorescence and flow cytometry techniques, they demonstrated a slight increase of PCNA in most G_1 cells as compared to G_0 cells. In late G_1, PCNA expression increased and peaked at the time of S phase. PCNA synthesis decreases to a level less than maximum in the G_2-M phases. Other investigators have used IHC to identify PCNA but did not discuss the cytologic distribution of PCNA expression in relation to phases of the cell cycle (13–16).

Immunohistochemical detection of PCNA is an alternative method for identifying replicating cells. Autoradiography procedures with tritiated thymidine and immunohistochemical techniques utilizing BrdU have been the methods of choice for analyzing S phase associated cells. Whereas tritiated thymidine and BrdU are restricted to S phase cells, the labeling index generated by PCNA encompasses various stages of cell replication, including S phase cells, and, therefore, should yield more information if scored correctly. Because it is an endogenous protein, demonstration of PCNA by IHC identifies cells in S phase at the time of tissue sampling, allowing measurement of cell replication at that time. Only cells exhibiting distinct (2+ or 3+) nuclear staining and no cytoplasmic staining should be scored as S phase cells to generate a labeling index. Labeling index comparisons using tritiated thymidine or BrDU vs PCNA have been studied by other investigators and show good correlation among the three methods (14,15).

In conclusion, PCNA was detected in formalin fixed,

paraffin embedded rat tissues by an improved biotin-streptavidin IHC procedure. Recognizably different patterns of staining are believed to correlate with the phases of the cell cycle (G_1, S, G_2, M, G_0). Studies are presently being conducted to investigate this hypothesis further. PCNA, as a naturally occurring cell marker of proliferating cells, offers an alternative method of investigating cell proliferation.

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