Innovative Methodology

Novel and quantitative DNA dot-blotting method for assessment of in vivo proliferation

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Submitted 14 October 2004; accepted in final form 3 November 2004

Ueda, Junji, Hiroshi Saito, Hiroaki Watanabe, and B. Mark Evers. Novel and quantitative DNA dot-blotting method for assessment of in vivo proliferation. Am J Physiol Gastrointest Liver Physiol 288: G842–G847, 2005. First published November 11, 2004; doi: 10.1152/ajpgi.00463.2004.—Immunohistochemical assessment of 5-bromo-2-deoxyuridine (BrdU) in tissue sections is a widely used method to evaluate cell proliferation in vivo. However, this method requires time-consuming preparation of paraffin sections and laborious counting of BrdU-labeled nuclei on multiple sections. Here, we report the development of a rapid and reliable method to quantitate BrdU incorporation in intestinal and liver tissues using a dot-blot method. In vivo models of colon or liver proliferation were used to analyze the usefulness and reliability of this new method. Mice were killed after BrdU injection, and genomic DNA was isolated from the tissues, denatured, and dot-blotted onto a nitrocellulose membrane. The incorporated BrdU was detected with a BrdU monoclonal antibody, and the signal intensity was densitometrically quantified. Results were compared with BrdU index determined by conventional immunohistochemistry on the same tissue samples. The patterns of colonic BrdU incorporation during fasting and refeeding, measured by the dot-blotting method and the immunohistochemical method, were similar. The BrdU incorporation in the regenerating liver after partial hepatectomy, evaluated by these two different methods, showed a strong correlation ($R^2 = 0.91, P < 0.01$). In addition, the inhibition of colon proliferation by the phosphoinositol 3-kinase inhibitor wortmannin was demonstrated by this dot-blotting method. In conclusion, the dot-blotting technique described in this report provides an accurate, more efficient, and possibly more reliable method for the assessment of in vivo proliferation compared with conventional immunohistochemical determination of BrdU-labeling index.

colon proliferation; liver proliferation; immunohistochemistry; novel method; fasting and refeeding; 5-bromo-2-deoxyuridine

THE MECHANISMS REGULATING proliferation in the gastrointestinal (GI) tract mucosa and liver are crucial questions that are currently being addressed by numerous clinical and basic science investigators from diverse backgrounds and disciplines (1, 6, 16). The GI tract mucosa is characterized by a rapid turnover (1, 17), which can be altered by various dietary perturbations, toxic agents, or other stimuli (2, 7, 18–20). For example, fasting results in a dramatic and rapid decrease of small bowel and colon proliferation; refeeding results in a marked proliferation of the gut mucosa (2, 7). Moreover, massive intestinal resection is associated with a marked adaptive hyperplasia of the remnant intestine (21). Similar to the intestine, loss of hepatic mass results in a dramatic regeneration of the remnant liver characterized by increased DNA synthesis of normally quiescent hepatocytes (5, 13). An accurate quantitation of proliferation in the GI tract and liver is crucial to the assessment of experimental manipulations or treatments that can enhance or inhibit growth.

Proliferation of tissue in vivo can be evaluated by DNA synthesis, which is commonly assessed by measuring the incorporation of either $[^3H]$thymidine or a thymidine analog 5-bromo-2-deoxyuridine (BrdU) (8, 12, 15). Briefly, the $[^3H]$thymidine or BrdU is administered in vivo, and incorporation of these compounds into tissue DNA is measured from tissues after death. The measurement of incorporated $[^3H]$thymidine is achieved by either autoradiography of the tissue sections or scintillation counting of the radioactivity in the isolated DNA from the tissues. However, this procedure is fraught with problems of handling and disposal of radioactive materials. Although immunohistochemical detection of BrdU does not use radioactive materials, this method requires time-consuming preparation of paraffin sections and laborious microscopic counting of BrdU-labeled nuclei from multiple sections. In addition, the objective counting of weak signals is difficult and may lead to spurious results.

Here, we report a novel method that allows for a rapid quantitation of BrdU incorporation in a large number of tissue samples. In this method, genomic DNA is extracted from tissues, denatured, and dot-blotted onto a membrane. The incorporated BrdU in the DNA is measured by an immunologic detection method similar to Western blot analysis followed by a quantitative densitometric analysis. Compared with standard BrdU immunohistochemical assessment, the new method reported in this study is highly reliable and quantitative. Therefore, this technique will be of value to investigators interested in the determination and accurate quantitation of proliferation in vivo.

MATERIALS AND METHODS

Mice and in vivo models of proliferation. Two-mo-old male C57BL/6 mice (Harlan, Indianapolis, IN) were acclimated at least 7 days in a 12:12-h light-dark cycle with free access to regular chow diet before experiments. All mice were injected intraperitoneally with BrdU (50 mg/kg body wt, Sigma, St. Louis, MO) for either 1 h (for the colon studies) or 3 h (for the hepatectomy study) before death. Mice were killed by cervical dislocation, and tissues were perfused with 30 ml of saline through the left ventricle into the open vena cava to wash out the circulating blood. Harvested tissues were snap-frozen in liquid nitrogen and stored at $-80^\circ$C until use. A small portion of the harvested tissue was fixed in 10% buffered formalin for immunohistochemical analysis.

For the fasting/refeeding study, mice were randomly divided into three groups: 1) control mice that were fed ad libitum, 2) fasting mice
that were fasted for 48 h, and 3) refeeding mice that were fasted for 48 h followed by refeeding ad libitum for 24 h. To investigate a role of phosphatidylinositol 3-kinase (PI3-K) in colon proliferation, the same fasting/refeeding model was used. After 48 h of fasting, mice were injected with either wortmannin (1.5 mg/kg body wt ip) or vehicle (2% EtOH) and were refeed ad libitum for 12 h. Control mice (fed ad libitum) and fasting mice (fasted for 48 h) were also included in this study. All mice were housed in wire-bottomed cages to prevent coprophagia.

For the hepatectomy study, all mice were anesthetized with halothane, and a midline laparotomy was performed after overnight fasting. All mice were randomly divided to two groups: 1) one group of mice underwent partial hepatectomy according to the procedure of Higgins and Anderson (10), and 2) control mice underwent an identical procedure that included laparotomy and liver mobilization without liver resection. All mice received saline (40 ml/kg body wt ip) to replace intraoperative fluid and lost blood and were killed at 48 h after operation.

A novel dot-blotting method for BrdU quantitation. To isolate genomic DNA from tissues, the frozen tissues were thawed, minced quickly with a razor blade, and incubated with DNA isolation buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.5% SDS) containing 100 μg/ml proteinase K (Promega, Madison, WI) overnight at 42°C. From this tissue lysate, genomic DNA was purified by two rounds of extraction with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1; pH 8.0) followed by precipitation with 1/10 volumes of 3 M sodium acetate (pH 4.8) and 2 volumes of ethanol. The precipitated DNA was washed twice with 80% ethanol and suspended in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) for 30 min at room temperature and kept on ice to prevent annealing. The DNA solution was placed on ice and neutralized by an equal volume of 1M Tris-HCl (pH 6.8). The single-stranded neutralized DNA (50 ng in 5 μl) was dot-blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and fixed by ultraviolet cross-linker Stratalinker (Stratagene, La Jolla, CA).

To visualize the BrdU signal, detection methods similar to Western blot analysis were performed. Briefly, the membrane was incubated with mouse anti-BrdU monoclonal antibody (1:2,000 dilution, B2531, Sigma) in buffer containing 20 mM Tris·HCl, pH 7.6, 136 mM NaCl, and 0.05% Tween 20 (TBS-T) containing 1% nonfat milk for 1 h at room temperature. Unlike Western blot analysis, a preceding blocking procedure to prevent nonspecific binding was not necessary. After being washed with TBS-T (10 min × 3 times at room temperature), the membrane was incubated with a horseradish peroxidase-conjugated anti-mouse IgG antibody (1: 5,000 dilution, Upstate, Lake Placid, NY) for 1 h at room temperature. The membrane was washed with TBS-T (20 min × 3 times, at room temperature), analyzed by enhanced chemiluminescence (ECL: Amersham, Arlington Heights, IL), and exposed to autoradiography film (Daigger, Vernon Hills, IL).

Intensity of each BrdU signal was quantitated by densitometric analysis in the sections was detected using BrdU Immunohistochemistry System (Oncogene, San Diego, CA) according to the manufacturer’s instructions. The sections were counterstained with hematoxylin. The BrdU-labeling index was determined by counting the number of BrdU-positive nuclei in at least 20 crypts of complete longitudinal sections in the colon sections and three high-power fields (>300 cells) in the liver sections and was expressed as a percentage of the number of labeled nuclei divided by the total number of nuclei.

Protein preparation and Western blot analysis. Tissues were homogenized in lysis buffer, protein concentrations were measured, and Western blot analysis was performed as described previously (4, 11). Briefly, equal amounts of protein were resolved on NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA) and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were incubated with anti-phosphorylated Akt (pAkt) antibody (9271, Cell Signaling, Beverly, MA) overnight at 4°C followed by secondary antibody conjugated with horseradish peroxidase. Membranes were developed using the ECL detection method. As a control, the membrane was reprobed with the antibody to Akt (9272, Cell Signaling).

Statistical analysis. Results are expressed as means ± SE. All statistical analyses were performed with StatView 5.0 software (SAS Institute, Cary, CA). Analyses between groups were determined by ANOVA. Post hoc ANOVA tests were determined by Fishers protected least significant difference. Analyses of the correlation between BrdU detection methods were determined by Pearson’s correlation test. A P value <0.05 was considered statistically significant.

RESULTS

Sensitivity and quantitation of the dot-blotting method for BrdU detection. To optimize the procedure of the dot-blotting method, initial experiments were performed to determine the best method of DNA denaturation and choice of membranes. As for the denaturation of the DNA, we found that this step is essential for the dot-blotting method. Without this step, no BrdU signals were detected. Although DNA denaturation was achieved by either alkaline solution or heating, the alkaline denaturation was preferred because of an easier handling and higher reproducibility. For immunohistochemical detection of BrdU, partial nucleolysis by pretreatment of tissue sections with hydroxycloride is required to make the target DNA accessible for the BrdU-antibody (3, 14). However, in the dot-blotting method, partial nucleolysis with HCl solution is not necessary, and in fact, partial nucleolysis significantly reduced intensity of BrdU signals. As for the selection of membranes, we tested a variety of membranes and found that nitrocellulose membranes gave the best overall results. Nylon membranes, which are frequently used for Southern and Northern blot analyses, gave unacceptably high background.

To examine the specificity and sensitivity of the dot-blotting analysis, various amounts of colon genomic DNA (0–200 ng) from BrdU-injected mice were dot-blotted onto nitrocellulose membranes, and the incorporated BrdU was measured as described in MATERIALS AND METHODS. As shown in Fig. 1, BrdU signal intensity increased in a linear fashion with an increasing amount of dot-blotted DNA, demonstrating that this method is applicable for quantitative analyses. In addition, this method is highly sensitive for detecting BrdU signals because as few as 13 ng of genomic DNA are required per dot. Furthermore, BrdU signals from each set of triplicate dots gave similar intensities, demonstrating that this dot-blotting method is highly reproducible. On the basis of these results, 50 ng of isolated DNA were dot-blotted in triplicate for each sample for the following experiments to assess the in vivo applications of this method.
Colonic BrdU incorporation in a fasting/refeeding model assessed by the dot-blotting method. We and others have previously shown (2, 7, 18) that prolonged fasting significantly decreases the proliferation of the intestinal epithelium and refeeding quickly stimulates the cell proliferation to normal or higher levels. We have used this fasting/refeeding model to examine whether the BrdU dot-blotting method can be applied to studies of intestinal proliferation. In this study, we compared BrdU incorporation in the colon of 1) mice after 48 h fasting, 2) mice after 48 h fasting followed by 24 h refeeding, and 3) mice fed ad libitum (n = 5 mice/group). The dot-blotting method and subsequent densitometric analysis showed that fasting decreased BrdU incorporation and refeeding increased BrdU incorporation to a level higher than the control. The mean BrdU incorporation was 100 ± 15, 10 ± 6, and 278 ± 98% in the control, fasting, and refeeding group, respectively (Fig. 2A). To compare the dot-blotting method with conventional BrdU-detection methods, we performed BrdU immunohistochemistry on paraffin sections from the same mouse colon samples. For each of the colon samples, BrdU-positive nuclei were counted under the microscope and the BrdU-labeling index was calculated as described in MATERIALS AND METHODS. The BrdU-labeling index was 100 ± 14, 4 ± 3, and 363 ± 81% in the control, fasting, and refeeding group, respectively (Fig. 2B). As shown in Fig. 2C, the patterns of BrdU incorporation measured by the dot-blotting method and the immunohistochemical method were similar. Taken together, we conclude that the dot-blotting method is as reliable as immunohistochemical calculation of labeled cells to measure BrdU incorporation in the intestinal mucosa. In addition, the dot-blotting method was more rapid and allowed for the quantitation of a large amount of tissue samples in a relatively short time.

A role of PI3-K/Akt pathway in colon proliferation assessed by the dot-blotting method. Previously, we have demonstrated a critical role for the PI3-K/Akt pathway in proliferation of the small bowel mucosa; treatment with the PI3-K inhibitor wortmannin (23) inhibited small intestinal proliferation following fasting and then refeeding (18). To investigate a role of the PI3-K pathway in colon proliferation, we analyzed the effect of wortmannin on BrdU incorporation using the dot-blotting method. For this study, we used the mouse fasting/refeeding model and examined whether administration of wortmannin affects the BrdU incorporation in the colon during refeeding. After 48 h of fasting, mice were injected with either wortmannin (1.5 mg/kg body wt ip) or vehicle (2% EtOH) and were re-fed ad libitum for 12 h. In addition, colons from mice fasted for 48 h and mice fed ad libitum without fasting were analyzed.
First, Western blot analysis of extracted protein from the colon was performed to examine the effect of wortmannin on the PI3-K/Akt pathway. Fasting reduced the level of pAkt, and refeeding with vehicle injection significantly increased pAkt expression; however, pAkt levels were not increased after refeeding in mice treated with wortmannin (Fig. 3A). These results confirm that wortmannin effectively suppresses PI3-K/Akt activation normally associated with refeeding after fasting.

Next, the colon proliferation was assessed by measuring BrdU incorporation using the dot-blotting method. Fasting resulted in a significant decrease in BrdU incorporation, and refeeding combined with vehicle injection increased the BrdU signal, which is consistent with our previous observation in Fig. 2. The BrdU incorporation of mice refed after fasting and treated with wortmannin was significantly less than that of mice refed and treated with vehicle (Fig. 3, B and C). These results demonstrate that wortmannin inhibited the increase of BrdU incorporation during refeeding and that, similar to the small bowel, the PI3-K/Akt pathway plays an important role in the rapid colon proliferation associated with refeeding.

Partial hepatectomy-induced BrdU incorporation assessed by the dot-blotting method. Liver regeneration is a well-known phenomenon characterized by a rapid tissue regeneration after loss of liver mass (5, 13). To evaluate the applicability of our dot-blotting method for assessing proliferation in a different tissue model, we measured the BrdU incorporation during hepatic regeneration after partial hepatectomy in mice. By the dot-blotting method, strong BrdU signals were detected from DNA extracted from the regenerating liver of mice compared with the DNA from the control sham-operated mice, indicating a vigorous tissue proliferation after the partial hepatectomy (Fig. 4A). These results were similar to the BrdU incorporation assessed by immunohistochemical counting of BrdU-labeling index (Fig. 4B). BrdU incorporation in the regenerating liver of mice after partial hepatectomy, evaluated by either the dot-blotting method or immunohistochemistry, showed a strong correlation. ($R^2 = 0.91, P < 0.01$; Fig. 4C). These results demonstrate that the dot-blotting analysis is a reliable method for analyzing proliferation of the liver as well as intestinal mucosa.

**DISCUSSION**

For analysis of in vivo proliferation, assessing BrdU incorporation in the tissue is a commonly used method. In the present study, we have developed a novel BrdU detection method in which tissue DNA is dot-blotted onto a nitrocellulose membrane, and the incorporated BrdU is immunochromatically quantified. We demonstrate the applicability of this technique in models of intestinal and liver proliferation. Although techniques have been reported for the nonquantitative detection of BrdU incorporation for bacterial screening (9, 22), our study is the first to describe a novel and sensitive method that allows for the accurate quantitation of BrdU incorporation in vivo.

This novel technique offers several advantages over the conventional BrdU-labeling index method that involves immunohistochemical BrdU detection and counting the BrdU-positive nuclei. First, a large number of tissue samples can be analyzed within 1 or 2 days. The use of commercial DNA isolation kits makes the entire process even more efficient. On the other hand, the traditional BrdU-labeling index method requires time-consuming processes such as tissue fixation, paraffin embedding, cutting tissue sections, immunohistochemistry, and laborious counting of BrdU-positive and -negative nuclei under the microscope. Second, whereas the dot-blotting method and the BrdU-labeling index analysis gave similar results, the dot-blotting method is probably more reli-
able than the BrdU-labeling index. Because tissue sections for BrdU-labeling index analysis are usually as thin as 5 μm, an immunohistochemical result from a single section is not entirely representative of the whole tissue. Therefore, multiple sections from different portions of the tissue must be analyzed for the BrdU-labeling index analysis. On the other hand, data from the dot-blotting method represent the average of the entire tissue. The third advantage of the dot-blotting method is a high objectivity. Immunohistochemical determination of BrdU-positive or -negative nuclei is sometimes ambiguous, particularly when there are many weakly stained nuclei. To circumvent this problem, more than one individual is sometimes required to count the BrdU-positive cells independently. Compared with the BrdU-labeling index method, the dot-blotting method gives objective results because it is quantitatively evaluated by densitometry. In addition, because this dot-blotting method does not use radioactive materials, it is safer and less laborious compared with the traditional evaluation of tissue proliferation using [3H]thymidine.

One disadvantage of the dot-blotting method, compared with the BrdU immunohistochemistry, is a lack of information regarding BrdU localization. Thus, if localization of BrdU incorporation is required, immunohistochemistry should be performed as a qualitative assessment. Quantitative data by the dot-blotting method would be obtained using the majority of the tissues, whereas, if necessary, confirmation of the BrdU localization on a few sections can be performed from the same tissue samples.

In the present study, we have shown that the dot-blotting method is useful for the quantitation of intestinal proliferation. We have recently reported an important role of the PI3-K/Akt pathway in the proliferation of small intestine in vivo (18); however, it is not known whether the PI3-K/Akt pathway also plays a role in normal colon proliferation. Our results demonstrate a strong correlation between colonic BrdU incorporation and pAkt levels; both are decreased during fasting and increased after refeeding. Furthermore, we demonstrate that wortmannin, a selective PI3-K inhibitor, decreased pAkt expression and colonic BrdU incorporation. These findings support the idea that PI3-K/Akt signaling regulates colonic epithelial cell growth in vivo and further identify the applicability of this technique to the assessment of intestinal proliferation.

In conclusion, we have developed a novel BrdU-detection method to evaluate tissue proliferation in vivo, which is safer than methods using radioactive thymidine and more rapid and reliable than immunohistochemical calculation of BrdU-labeling index.
ing index. We confirmed that this method is useful for the quantitative assessment of intestinal and liver proliferation. Moreover, this technique should have broad applications for the analysis of proliferation in other tissues given the sensitivity of BrdU detection.

ACKNOWLEDGMENTS

The authors thank Karen Martin and Eileen Figueroa for assistance with manuscript preparation.

GRANTS

This work was supported by National Institutes of Health Grants RO1-DK-48498, R37-AG-10885, and PO1-DK-35608 by the John Sealy Memorial Endowment Fund at the University of Texas Medical Branch.

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