Quantification of Proliferating Cell Nuclear Antigen in Large Intestinal Crypt by Computer-assisted Image Analysis

Hsi-chiang Lin, Alexandre V. Sotnikov, Lisa Fosdick, Robert M. Bostick, and Walter C. Willett

Departments of Molecular & Cellular Toxicology [H. C. L.], Epidemiology [W. C. W.], and Nutrition [H. C. L., W. C. W.], Harvard School of Public Health, and the Channing Laboratory, Department of Medicine, Harvard Medical School, and Brigham and Womens Hospital [W. C. W.], Boston, Massachusetts 02115; Pathology Expertise, Inc., Newton, Massachusetts 02165 [A. V. S.]; University of Minnesota, Minneapolis, Minnesota 55454 [L. F.]; and Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27157 [R. M. B.]

Abstract

Measurements of proliferating cell nuclear antigen (PCNA) labeling of the large intestinal crypts scored by experienced observers were compared with those generated by computer-assisted image analysis (CAIA). (CAIA was performed at Pathology Expertise, Inc., Newton, MA, by A. V. Sotnikov.) Serial sections (3 μm) of the rectal biopsy specimens from 32 patients were immunostained for PCNA and then counterstained with hematoxylin. The same set of slides and rules for the location/acquisition of complete crypts was used to assess a minimum of ten complete crypts/patient. Each crypt was subdivided longitudinally into five equal compartments. With CAIA, the images were stored digitally, and once the color references were set, the areas occupied by labeled and counterstained nuclei were quantified automatically. The labeling index (LI) was calculated from the PCNA-labeled nuclei area/total nuclei area in CAIA and from the number of labeled cells/total number of cells in visual scoring. The LI of whole crypts averaged 1.04 ± 0.18 by CAIA and 3.91 ± 0.46 by the visual method, and the Spearman correlation (rS) between the two methods was 0.89. The different modes of evaluation and color reference selection are likely to have contributed to the differences in the LI ranges observed in the two methods. The high correlation between PCNA quantification by CAIA and visual scoring by experienced technicians indicates that CAIA can reliably rank individual subjects. Thus, measurement of PCNA labeling by CAIA is a practical alternative for evaluating colorectal epithelial cell proliferation.

Introduction

Cell proliferation has often been studied as a biomarker for cell growth in carcinogenesis studies (1–11). Traditionally, the incorporation of tritiated thymidine or its analogue bromodeoxyuridine into DNA during the synthesis phase of the cell cycle has been used as a measure of proliferation. Several monoclonal antibodies to PCNA, which show peak expression at the late G1-S phase, have been developed to identify the proliferating cells (6–7) and have shown to be comparable to tritiated thymidine or bromodeoxyuridine incorporation as reliable indicators for cell proliferation (9, 10). The immunohistochemical method of detecting PCNA has the advantage of (a) avoiding the administration of labeling compounds; and (b) being used for both fixed embedded and frozen tissues. These features are especially appealing in the retrospective study of archival materials, in screening for people at risk, or in chemopreventive trials.

Visual quantification of labeled versus unlabeled cells is a tedious and time-consuming process, and the results obtained for the same slides evaluated at different time periods by observers have shown large inter- and intra-observer variability (12, 13). Thus, multiple observations of a single slide by several experienced observers have been utilized routinely to minimize error. For epidemiologists who require a simple, reliable method to study large numbers of samples in the population, the use of computers to quantify cell proliferation status would be very useful (8, 14–17). CAIA has been recommended to increase objectivity, reliability, and reproducibility in the assessment of biomarkers and was used in the measurement of PCNA labeling in brain tumor (18), breast carcinoma (19) and leiomyoma cells (20). With the advent of affordable high resolution red/green/blue, charge-coupled device camera system video cameras, the present study was undertaken to evaluate the use of computer-assisted true color image analysis in quantifying PCNA. The baseline biopsy slides from a sample of 32 patients in the Calcium and Colorectal Epithelial Cell Proliferation Trial (20), carried out at the University of Minnesota (Minneapolis, MN), were sent to the Harvard School of Public Health (Boston, MA), for the semiautomated image analysis.

Materials and Methods

Patient Population. Participants were recruited for the Calcium and Colorectal Epithelial Cell Proliferation Trial (21) from the patient population attending a private practice gastroenterology group that performs approximately 60% of all colonoscopies in the Minneapolis-St. Paul (MN) area. To be eligible for the study, subjects must have been 30–74 years of age with normal or spontaneously regressed colorectal polyps and without colorectal adenomas for the previous 10 years. Colonoscopies were performed at the University of Minnesota Medical Center, the University of Minnesota Medical Outpatient Center, and the Mayo Clinic. The baseline biopsy slides from 32 patients were reviewed and scored by one of the authors (A. V. S.). For the study, the LI was quantified by CAIA and visual scoring.

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2 To whom requests for reprints should be addressed, at Harvard School of Public Health, Department of Molecular & Cellular Toxicology, 665 Huntington Avenue, Boston, MA 02115.

3 The abbreviations used are: PCNA, proliferating cell nuclear antigen; CAIA, computer-assisted image analysis; LI, labeling index; rS, Spearman correlation coefficient; LC, labeled cell number; Phi40, fraction of labeling in the upper 40% of crypts; Phi(c)40 for visual method and Phi(a)40 for CAIA.
Computer-assisted Image Analysis of PCNA

...age, in general good health, and capable of informed consent. Additional criteria for patient selection included: (a) a history of pathologically confirmed adenomatous polyps within the previous 5 years; (b) consumption of a Western-style diet; (c) no contraindications to calcium supplementation or rectal biopsy procedures; and (d) no medical conditions, habits, or medication usage that would otherwise interfere with the study.

Biopsy. The rectal biopsy procedure was performed without the subject’s first taking a laxative, enema, or other bowel-cleansing preparation. Three biopsies that appeared grossly adequate were taken from normal-appearing mucosa of the rectum 10 cm above the level of the anus. The biopsies were placed in a culture medium immediately. Within 15 mm, the biopsies were stretched out flat, lumen side up on bibulous paper; the biopsies were fixed in 70% ethanol. After the biopsies were fixed in 70% ethanol and processed for 5 h in a tissue processor, two biopsy specimens/patient were embedded in one paraffin block. Biopsies were embedded, positioned, and cut with a microtome so that crypts were longitudinally sectioned from base to lumen. Section levels were 3-μm thick and taken 50 μm apart (taking sections 50 μm apart precludes scoring the same crypt more than once while maximizing tissue yield). Sections were then mounted on microscope slides. For each biopsy visit, four slides, each containing five section levels, were prepared.

Immunostaining. Slides were deparaffinized and rinsed in PBS. The slides were then mounted on a sequenza stainer (Shandon) and received three 5-min washes with PBS (pH 7.4). Three drops of a blocking agent, 5% normal horse serum (Vector S-2000; diluted in PBS), were applied for 20 min, and then three drops of PC-10 clone PCNA-antibody (Oncogene NA03; diluted 1:200 with PBS) were added for an additional 45 min. The slides then received three 5-min washes with PBS, and three drops of the secondary antibody, rabbit anti-mouse IgG (DAKO E413; diluted 1:200 with PBS), were applied for 45 min. The slides again received three 5-min washes with PBS, and the endogenous peroxide was blocked with 0.3% H2O2 for 10 min. After three additional 5-min washes with PBS, three drops of the tertiary agent, avidin-biotin complex (Vector Standard Kit PK-6100 at one-half strength), were applied for 45 min. The slides received three additional 5-min washes with PBS, then were developed by immersing them with the chromogen, 3,3′diaminobenzidine (Sigma D-9015), in a 1 mg/ml solution with 0.003% H2O2 for 2–5 min while monitoring the chromogenic development by using light microscopy. Slides were then rinsed with distilled water, counterstained with Harris’s hematoxylin, dehydrated, and coverslipped. All slides from a single participant were batched with a negative control slide and a human tonsil positive control slide.

Rules for Scoring. Colon crypts longitudinally sectioned from base to lumen were analyzed. To obtain a comparable and reliable evaluation was performed using PEI ProStation (Pathology Expertise, Inc., Newton, MA). The histological sections were viewed by an observer (A. V. S.) with an Olympus BHTU microscope (Olympus Corp. Precision Instrument Division, Lake Success, NY). The images of at least ten complete crypts/patient were digitized at ×200 using a high resolution red/green/blue, charge-coupled device camera system video camera and archived as digital specimens. After performing the digital subtraction of the the lamina propria and the lumen, each crypt was divided into five equal compartments using a semi-
automated subroutine. The thresholds for labeled and counterstained nuclei were tested and adjusted for sensitivity and specificity to assure the accurate and precise measurement of the biomarker throughout the entire set of digital specimens.

Data Analysis. For quality assurance, two color references or thresholds for labeled nuclei were defined by the observer: low and high. The high threshold was used to measure only the strong labeling (relatively narrow range of intense brown coloration). The low threshold was used to measure the entire labeling (relatively wide range of weak to intense brown coloration). A single, third color reference was developed to measure the total surface area of both labeled and counterstained nuclei. All data acquisition using CAIA was completed without knowledge of the visually obtained values.

The overall cell proliferation, LI, was calculated in the visual method for each biopsy by dividing the total number of LCs by the total number of cells counted from all scored crypts from the biopsy and multiplying by 100 ([100 × LC/total number of cells counted]). Crypts were subdivided horizontally by cell count height into five equal-sized compartments (3). A measure of the distribution of proliferating cells in the crypt, the proportion of proliferating cells that were in the upper (luminal) 40% of the crypt, was calculated on each biopsy by dividing the number of labeled cells counted in the upper 40% of the crypt (LCIV–V) by the total number of LCs and multiplying by 100 ([100 × LCIV–V/LC]). In CAIA, LIs for the whole crypt and for each of the five compartments were computed by measuring and relating labeled and total nuclear area (18) rather than using nuclei numbers. Phi(a)40s were similarly computed. Data are expressed as mean ± SEM. Correlation coefficients comparing LIs obtained from the visual and CAIA methods were calculated for each compartment and for the whole crypt. Reader reliability was analyzed using intraclass correlation coefficients.

Results

Fig. 1, a-f show the processes of obtaining PCNA and nuclear labeling information from one of the sample slides via CAIA. Two levels of color references were used for PCNA labeling, the high threshold quantifying only the dark brown-labeled nuclei (Fig. 1c). A strong LI was calculated from the information obtained using the high threshold (dark brown), whereas weak LIs were calculated from those using the low threshold (both light and dark brown). Fig. 2 showed the whole crypt LIs of the 32 specimens calculated from the information obtained from these different color references for PCNA staining. The Spearman correlations between the strong and weak whole crypt LIs were 0.84 and 0.80 for the CAIA and the visual method, respectively (Fig. 2). With the visual method, from the full Calcium and Colorectal Epithelial Cell Proliferation Trial, the slide scoring intra- and interreader reliabilities (intraclass correlation coefficients), using the strong labeling were 0.91 and 0.77 for LI and 0.77 and 0.79 for Phi(a)40, respectively. Using all labeled cells (strong plus weak) the corresponding reader reliabilities were 0.92, 0.76, 0.90, and 0.78.

Both strong and weak LIs for each of the five compartments of the crypt and the whole crypt were shown in Fig. 3, a and b. The overall LIs obtained from the CAIA were much lower than those obtained from the visual method: the CAIA:visual method ratio varied from 0 to 0.8. Nonetheless, both methods showed major differences between LIs in the upper and lower crypt compartments, with the major DNA replication zone located at the lower one-half of the crypt.

The correlations between LIs measured by the visual and CAIA methods for each of the five compartments, the whole crypt, and Phi 40s (fraction of labeling in the upper 40% of crypts) are shown in Fig. 4 and Table 1. The overall LIs for
strong labeling were 1.04 ± 0.18 by CAIA and 3.91 ± 0.46 by the visual method ($r_s = 0.89$ between the two methods). The overall LIs for weak labeling were 3.70 ± 0.47 by CAIA and 15.23 ± 1.57 by the visual method ($r_s = 0.73$ between the two methods). The correlations between the visual method and CAIA decrease as compartments shift toward the lumenal side of the crypt. However, there is a considerable good correlation ($r_s = 0.72$) between Phi(a)40 (2.28 ± 0.63) in the CAIA and Phi(c)40 (5.05 ± 1.32) in the visual method.

**Discussion**

CAIA has been shown to greatly increase the accuracy, sensitivity, and reproducibility of various assays that use chromogenic probes to measure the fraction of cycling cells or cells in S phase. In the assessment of cell proliferation by CAIA, Sallinen et al. (18) reported that an area-related PCNA index was more reproducible than a cell number-related PCNA index. Thus, in the present study, we chose to use the area-related PCNA index in CAIA. We followed strict scoring rules in crypt selection and evaluated the correlation between CAIA quantification and the visual scoring of PCNA. In general, to minimize the inter- and intra-observer variation in visual assessment of PCNA, highly trained observers are necessary, and they must score many crypts per specimen (12, 13). We took the mean values obtained by the two observers using visual scoring method to correlate with those obtained by CAIA. The correlation coefficients of whole crypt LIs and Phi40 between the
two methods were 0.89 and 0.72, respectively, for strong labeling and 0.73 and 0.61, respectively, for weak labeling.

LIs obtained from the CAIA were lower than those from the visual method (Table 1; Fig. 3). Threshold settings have been reported to contribute about 5% of the variability in the overall reliability in quantifying PCNA (12). In CAIA we obtained 4% variability in setting color references. The higher overall reliability in quantifying PCNA (12). In CAIA we obtained 4% variability in setting color references. The higher overall reliability in quantifying PCNA (12). In CAIA we obtained 4% variability in setting color references. The higher overall reliability in quantifying PCNA (12). In CAIA we obtained 4% variability in setting color references. The higher overall reliability in quantifying PCNA (12).--served by both methods in 8 of 32 specimens, the visual method

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LIs obtained from the CAIA were lower than those from the visual method (Table 1; Fig. 3). Threshold settings have been reported to contribute about 5% of the variability in the overall reliability in quantifying PCNA (12). In CAIA we obtained 4% variability in setting color references. The higher values reported by the visual method are likely to be explained by the different modes of evaluation in CAIA and the visual method. In CAIA the relative “positivity” of the nucleus is calculated by directly quantifying and relating the negative and the positive areas of the nucleus. Thus, nuclei with one-half labeling are reported as having 50% positivity. On the other hand, the visual method requires a complex cognitive process involving both discrimination (yes/no) on whether a nucleus is labeled and a qualitative judgment (grading) meant to compensate for the crudeness of discrimination. It is also possible that the emphasis on positive cells may lead to a biased perception of their importance and exaggerated counts.

Indeed, in a highly tiresome and time-consuming visual

scoring process, it can be difficult for a person to maintain a consistent threshold for a color reference while scoring over 100 cells/crypt column from the opening in the lumen down to the base of the crypt and up again to the lumen. Humans may have a tendency to look for positive signals, especially in the upper crypt compartments with a few, if any, labeled nuclei present, thus potentially exaggerating the scores. In the uppermost compartment V, where neither method showed any PCNA-labeled nuclei for 25 of 32 specimens, considerable compartment V LIs were observed in the 7 other specimens with the visual method, whereas no or near zero labeling was obtained with CAIA for 5 of the remaining 7 specimens. In compartment IV, where no PCNA-labeled nuclei were observed by both methods in 8 of 32 specimens, the visual method also had a higher tendency to show labeling, whereas CAIA had no or near zero labeling. The difference between the visual and computer-generated data is indicative of the difficulties faced by an observer during the visual evaluation of immunolabeling in specific tissues compartments. The fact that CAIA and visual LIs correlate quite well toward the lower compartments, containing numerous distinctively labeled nuclei, suggests that human observers may be more consistent when labeling is unequivocal. However, because the elevations of upper crypt LIs or the expansion of proliferative compartments have been shown to be related to carcinogenesis (2, 22, 23), the reproducibility for the sparsely labeled upper compartment is extremely important. In CAIA, the thresholds are developed after measuring strong labeling indices for large intestinal crypts. Phi 40s (mean ± SEM; n = 32), the fraction of labeled cells or nuclei present in the upper 40% of the crypt, for both methods are also shown.

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fication by CAIA and visual scoring by experienced technicians indicates that CAIA is a reliable method for ranking individual subjects. Measurement of PCNA labeling assisted by computer is a practical and, perhaps, superior alternative for evaluating colorectal epithelial cell proliferation in epidemiological studies.

References

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