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In vivo measurement of DNA synthesis rates of colon epithelial cells in carcinogenesis

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Abstract

We describe here a highly sensitive technique for measuring DNA synthesis rates of colon epithelial cells in vivo. Male SD rats were given ^2H_2O (heavy water). Colon epithelial cells were isolated, DNA was extracted, hydrolyzed to deoxyribonucleosides, and the deuterium enrichment of the deoxyribose moiety was determined by gas chromatographic/mass spectrometry. Turnover time of colon crypts and the time for migration of cells from basal to top fraction of the crypts were measured. These data were consistent with cell cycle analysis and bromodeoxyuridine labeling. By giving different concentrations of a promoter, dose-dependent increases in DNA synthesis rates were detected, demonstrating the sensitivity of the method. Administration of a carcinogen increased DNA synthesis rates cell proliferation in all fractions of the crypt. In conclusion, DNA synthesis rates of colon epithelial cells can be measured directly in vivo using stable-isotope labeling. Potential applications in humans include use as a biomarker for cancer chemoprevention studies.

Keywords: Aspirin; Azoxymethane; Colon; Deuterated water; DNA; Gas chromatography–mass spectrometry

The evolution of colon cancer is believed to follow the multi-stage carcinogenesis model [1]. At all stages of colon carcinogenesis, increased cell proliferation and expansion of the proliferative zone toward the surface of crypts have been observed in human and animal studies [2–6]. Increased colon epithelial cell (CEC) proliferation plays a central role in the evolution of colon carcinogenesis [5,7,8].

Although many biomarkers related to cell proliferation have been used to detect susceptibility to colon cancer, there currently exists no established technique for measuring CEC proliferation in vivo that is applicable in humans and is without toxicities. Measurement of DNA synthesis is generally accepted as the most direct biochemical index of cell proliferation [9]. We recently developed a new method for measuring DNA synthesis rates in vivo that does not involve radioactive or mutagenic reagents [9–11]. This technique allows measurement of new DNA synthesis by use of stable isotope labeling with mass spectrometric analysis.

The principle of the method is as follows. During ^2H_2O (heavy water) administration, dividing cells incorporate deuterium into the covalent C–H bonds of the deoxyribose (dR) moiety of deoxyribonucleotides, which are the precursors for DNA synthesis (Fig. 1A). Non-dividing cells do not incorporate deuterium into dR. Newly divided cells therefore can be identified based on the deuterium isotopic enrichment in dR in DNA. This method has several advantages over traditional cell proliferation assays [9–12]. In particular, ^2H_2O labeling provides a dynamic measurement with a potentially long window of observation compared to static measures (e.g., Ki67 or proliferating cell nuclear antigen

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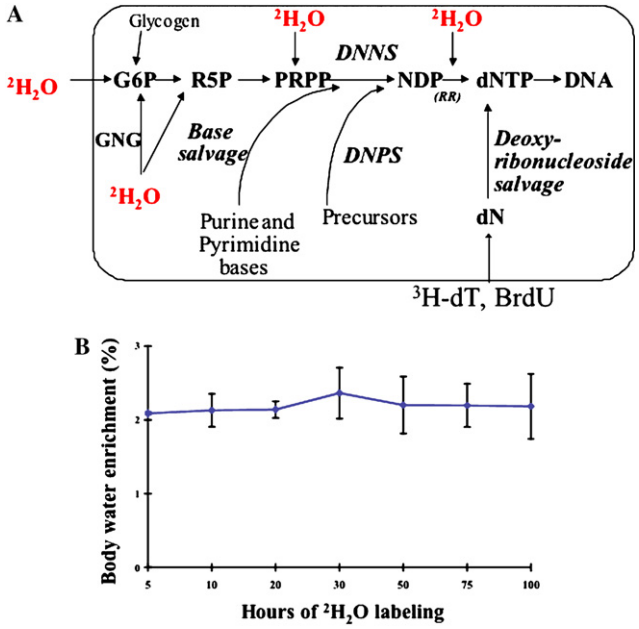

Fig. 1. (A) Pathways for labeling DNA with ^2H_2O in cells. *Abbreviations:* G, glucose; GNG, gluconeogenesis; P, phosphate; R, ribose; DNPS, de novo purine synthesis pathway; DNNS, de novo nucleotide synthesis pathway; NDP, nucleoside diphosphate; RR, ribonucleoside reductase; dNTP, deoxyribonucleoside triphosphate; DNA, deoxyribonucleic acid; dN, deoxyribonucleosides; [^{3}H]dT, tritiated thymidine deoxyribonucleoside; and BrdU, bromodeoxyuridine. (B) Body water enrichments during intake of 4% ^2H_2O in drinking water (measurements from 5 h to 4 days are shown). Values shown are means ± SD.

expression) or ex vivo measurements (e.g., tritiated thymidine ([^{3}H]dT) or bromodeoxyuridine (BrdU) labeling of biopsied tissues [13]). Also, endogenous ^2H labeling of purine deoxyribonucleotides in DNA through the de novo nucleotide synthesis pathway (Fig. 1A) is more constant and less affected by extracellular nucleoside contribution than is the salvage pathway used by [^{3}H]dT and BrdU. Moreover, stable isotopes are not radioactive or mutagenic and are therefore safe and applicable for use in humans [9,11].

Epidemiologic studies show that non-steroidal anti-inflammatory drugs (NSAIDS), including *N*-acetyl-salicylic acid (aspirin), protect against colorectal cancer [14]. NSAIDS are known as strong inhibitors of aberrant crypt growth in the promotional stage of colon cancer [15]. The mechanism of protection has been proposed to be inhibition of a cyclo-oxygenase 2 (COX2) pathway [14]. Other possible mechanisms have also been suggested [16].

Here, we describe the use of a stable isotope (^2H_2O) mass spectrometric technique for measuring DNA synthesis rates of CEC in vivo. The CEC proliferative responses to a promoter, a carcinogen, and a chemopreventive drug are also established. This method may prove useful as a biomarker of colon cancer in humans. Portions of this work have been presented previously in abstract form [17,18].

Materials and methods

Animals. Adult Sprague–Dawley rats (male, 4–7 weeks old, Simonsen, San Jose, CA) were used. All procedures were approved by the UC Berkeley Office of Laboratory Animal Care. Housing was 3 rats per cage. Diet was Purina rat chow, provided ad libitum. A 12 h light:dark cycle was maintained. At each measured time point, rats were euthanized by carbon dioxide asphyxiation. There were no significant changes in the body weights of animals while they were given ^2H_2O, cholic acid (CHA) diet or BrdU treatment. With CHA diets, watery stools were observed.

For the azoxymethane (AOM) and aspirin studies, male CD rats were purchased at 6 weeks of age from Harlan Sprague–Dawley. At 7 weeks of age, all rats were injected (i.p.) with saline or AOM (15 mg/kg body weight in saline) twice a week for two weeks. The next day, diets with aspirin or chow were started. Heavy water was then given during the last 3 days of diet administration.

Diets. Diets containing different concentrations of CHA (0, 0.1, 0.3, and 0.5%) modified from AIN 93 G (Madison, WI) were custom made and purchased from Harlan Teklad. The diets were given for 3 weeks. Diets were stored in a refrigerator and changed every two days.

Diets with aspirin (Sigma, 0.4 g/kg diet, Dyets) or chow were premixed into pellets by the manufacturer and were given for 10 days. Diets were changed every 3 days.

^2H_2O administration and body water enrichments. Rats were injected with 100% ^2H_2O (Cambridge Isotopes, Andover, MA) i.p. to achieve 2.0–2.5% enrichment in body water, assuming 60% of their body weight as water, followed by 4% ^2H_2O administration in drinking water ad libitum throughout the study [11]. The 4% enrichment of ^2H_2O in drinking water was chosen because it produces sufficient enrichments in DNA from colonocytes and has no known toxicities. For the AOM studies, rats were injected with 100% $2H_2O$ i.p. to achieve 5% body water enrichment, followed by 8% $2H_2O$ in drinking water.

Enrichment of ^2H_2O in body water (blood) was measured by a new GC/MS technique, after chemical conversion to tetrabromoethane [10,11]. Briefly, the hydrogen atoms in H_2O were transferred to acetylene by addition of 2–5 μl water via syringe to a chip of calcium carbide in a sealed vial, equipped with a 3 ml syringe inserted into the septum. The resulting acetylene gas was drawn into the 3 ml syringe and expelled into another sealed vial containing 0.5 ml Br_2 (0.1 mM) dissolved in CCl_4. After 2 h of incubation at room temperature, the remaining Br_2 is reacted with cyclohexene dissolved in CCl_4 (10% solution). This solution is injected into the GC/MS for analysis. GC/MS analysis was performed with a DB-225, 30 m column at 220 °C, using methane chemical ionization with selected ion monitoring. The $C_2H_2Br_3^+$ fragment (*m/z* 265 and 266, representing the M_0 and the M_{+1} ion of the $^{79}Br^{79}Br^{81}Br$ isotopologue), was used for calculating ^2H enrichment, by comparison to standard curves generated by mixing 100% ^2H_2O with natural abundance H_2O in known proportions [10].

BrdU administration. BrdU immunohistochemistry studies were performed after 3 weeks of CHA diets (0–0.5%). Sprague–Dawley rats received an i.p. injection of BrdU (Sigma) at 160 mg/kg body weight in dimethyl sulfoxide (final concentration 100 μg/ml) two hours before sacrifice.

Isolation of CEC from colon tissue. Colon was excised fresh at necropsy. CECs from different zones of the crypt were isolated using the non-enzymatic, mechanical dissociation method of Brasitus [19,20]. Briefly, CECs from different crypt zones (basal proliferative zone and top mature zone) were collected by incubating the colon sac filled with PBS buffer with chelating agents in an Erlenmeyer flask, also filled with PBS at 37 °C. The tissue was shaken in a 37 °C water bath for 20 min, then 15 and 30 min for collecting CECs from top, middle, and the basal zones, respectively. The middle zone (transitional CECs) was discarded except for AOM studies. The collected CECs were then applied to a discontinuous Percoll gradient. Forty-five percentage Percoll was overlaid on 75% Percoll (Amersham Biosciences, Uppsala, Sweden, [21]) and centrifuged to separate CEC from intraepithelial

lymphocytes, fecal debris, and other cell types. Viable colon epithelial cells were obtained from the top of the 45% Percoll gradient after the centrifugation.

Isolation of deoxyadenosine from DNA and derivatization of dR. Genomic DNA from colonocytes was isolated using a Qiagen kit (Qiagen, Valencia, CA). DNA was enzymatically hydrolyzed to free deoxyribonucleosides as previously described [10,11]. For separation of deoxyadenosine (dA) from the other deoxyribonucleosides, an LC18 SPE column (Supelco, Bellefone, PA) was used. The column was washed with 100% methanol (2 ml) and water (2 ml). The hydrolyzed DNA sample was then added to the column and nucleosides other than dA were eluted with five washes of H_2O (five times of 1 ml washes). The dA was then eluted with 50% methanol (1 ml) as previously described [10]. The isolated dA was cleaved of the base moiety (by overnight shaking with cation exchange resin (Bio-Rad, Hercules, CA)) to obtain dR. The pentose-tetraacetate (PTA) derivative of dR was synthesized and isotopic enrichment was determined by selected ion monitoring of *m/z* 245 and 246, representing M_0 and M_{+1} masses, respectively [10]. For the AOM study, perfluorotetraacetate (PFTA) derivatives of dR was synthesized, with selected ion monitoring of *m/z* 435 and 436, representing M_0 and M_{+1} masses, respectively.

GC/MS analysis was performed by methane CI, using a 30 m DB-225 column (0.25-mm id, 0.25-μm film thickness, J & W Scientific, Folsom, CA). The derivatives that we analyze contain only the dR moiety, not the base portion, of purine deoxyribonucleosides, so label incorporation into the base moiety is not a confounding factor [9–11].

Calculations. Unlabeled (representing natural abundance) dA standards were analyzed in each GC/MS run to establish the dependence of measured isotopic ratios on the amount of sample injected (abundance sensitivity [11]). This dependence can be characterized by plotting the abundance of the parent M_{+0} ion (*m/z* 245) versus the ratio of M_{+1} to M_{+0} plus M_{+1} ions (246/(245 + 246)). A linear regression of the ratio versus M_{+0} abundance was calculated, as described previously [11]. The regression line was then used to calculate the natural abundance ratio at any particular M_0 abundance [11], for calculation of excess abundances in samples.

The fraction of newly synthesized DNA (*f*, fractional synthesis) was calculated by use of the precursor–product relationship. The isotopic enrichment of fully (or nearly fully) turned-over CECs represents the true precursor enrichment (i.e., maximum enrichment) for CECs [11]. To obtain fully turned-over CECs separate group of rats ($n = 8$) was maintained on ^2H_2O for 2–3 weeks for use as a comparison (denominator) or a maximum value [9–11] and analyzed concurrently with experimental samples. Body water enrichment from 2 to 3 week labeled rats attained a constant level after 6 days [22]. The fraction of new cells produced (*f*) during each time point was determined by the following equation.

$$\mathrm{EM_1} = \frac{\text{Abundance of } M_{+1}\text{(sample)}}{\text{Abundance of } M_{+0} + M_{+1}\text{(sample)}} - \frac{\text{Abundance of } M_{+1}\text{(unlabeled standard)}}{\text{Abundance of } M_{+0} + M_{+1}\text{(unlabeled standard)}},$$

$$f(\text{fraction of new cells}) = \frac{\mathrm{EM_1(CEC)}}{\mathrm{EM_1(fully\ replaced\ CEC)}},$$

where M_{+0} represents parent mass isotopomer of derivatized dR; M_{+1}, mass plus one isotopomer of derivatized dR; and EM_1, excess abundance of M_{+1} mass isotopomer. Production rate of new cells was calculated by dividing the fraction of new cells produced at each time point by the duration of the ^2H_2O labeling period (days).

BrdU immunohistochemistry. The distal end of the colon (1 cm) was cut, fixed in 37% phosphate-buffered formalin (Fisher) for 15 h, transferred into cold 70% ethanol, paraffin embedded, sliced into 5 μm sections, and mounted onto slides. Slides were stained with anti-BrdU monoclonal antibody (1:50 dilution in PBS) (Becton–Dickinson, San Jose, CA) according to the method of Murrill et al. [23]. Cells were counterstained with Mayer's hematoxylin solution (Sigma) and slides were preserved with Permount (Fisher Scientific). The labeling index [(no. of BrdU positive cells)/total no. of cells counted] was determined using a Zeiss microscope with a video camera and a monitor. At least 10–15 well-defined crypts (1000–1500 total cells) were counted from each animal. All cell counts were carried out independently by two observers blinded to the study design.

Cell cycle analysis using propidium iodide staining. CECs were fixed and permeabilized in the same way as for the BrdU staining. The cells were stained according to the method of Potten et al. [24]. Propidium iodide (PI) (50 μg/ml) in PBS (1 ml) was added to the cells, with incubation for 3 h at room temperature. The cells were analyzed by a Beckman-Coulter EPICS XL flow cytometer (argon ion air-cooled laser, emission at 488 nm/15mW power). Histograms were analyzed using WynCycle (Phoenix Flow Systems, San Diego, CA) software.

Confirmation of sequential isolation procedure for CEC from tissue. Cells were fixed in 1% paraformaldehyde for 20 min and incubated with fluorescein-labeled Dolichos Biflorus Agglutinin (DBA, Vector Labs, Burlingame, CA, 40 μg/ml) for 4 h at 4 °C. Cells were then washed with PBS two times before analyses. Negative controls were incubated with 200 mM *N*-acetylgalactosamine to inhibit the binding of DBA to colonocytes followed by incubation with fluorescein-labeled DBA.

Statistical analyses. One-way ANOVA (results were considered significant at $p < 0.05$), followed by a Tukey's HSD (honestly significant difference) test to determine differences among the groups. Correlation coefficients were calculated using Spearman's correlation equation.

Results

Constancy of deuterium enrichments in body water during the ^2H_2O labeling period

Body water enrichments of deuterium were measured serially (Fig. 1B). On 4% ^2H_2O in drinking water, body water ^2H_2O enrichment reached plateau by 5 h and remained at the steady state ranging from 2.1% to 2.4% in rats. We have shown elsewhere that longer administration of ^2H_2O for up to 11 weeks results in relatively constant body water enrichments over time in individual rats [11].

Turnover rate of CECs from colon during ^2H_2O labeling

It is known that CECs divide in the proliferative zone of the crypt and, as they differentiate, migrate up the crypt to the mature zone until they are sloughed off [25].

The turnover rate of DNA from CECs and time lag of CEC progression from the basal to the top crypt zones were measured using ^2H_2O administration for 2, 4, 6, 8, and 10 days. The fraction of new cells produced (*f*) in the proliferative (basal) zone increased from 33 ± 4% to 71 ± 7% and 98 ± 10%, at days 2, 4, and 6, respectively (mean ± SD, Fig. 2B). The values at days 6, 8, and 10 (98 ± 10%, 97 ± 3.5%, and 99.7 ± 0.5%) were not statistically different, meaning that *f* reached its plateau at day 6 and the proliferative zone was fully replaced with new cells after 6 days. The fraction of new cells in the mature (top) zone was 15.8 ± 10%,

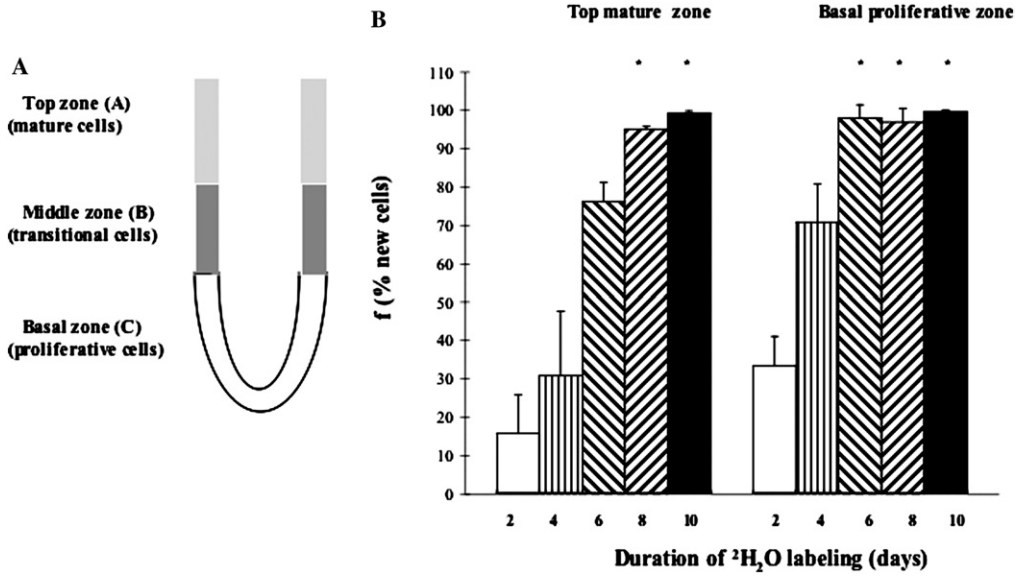

Fig. 2. (A) Schematic model of the colon crypt divided into three zones: basal (proliferative zone), middle (transitional zone), and top (mature zone). (B) Turnover rate of CECs during ^2H_2O labeling ($n = 3$ for each time point). Rats received 4% ^2H_2O drinking water for 2–10 days, after a priming dose. *, one-way ANOVA ($p < 0.05$ vs. day 2 value in each zone). Values shown are means ± SD.

31 ± 17%, 76 ± 5%, 95 ± 8%, and 99 ± 0.66% at days 2, 4, 6, 8, and 10, respectively. The values at days 8 and 10 were not statistically different. Thus, the mature zone was replaced with new cells after 8 days. The time lag for cell migration from the proliferative to mature fraction was therefore estimated to be approximately 2 days, based on the lag-time between complete cell replacement in the proliferative (6 days) and the mature (8 days) zones (Fig. 2B).

Fig. 3 shows that the protocol that we used successfully separates CECs from the top and basal zones of the crypt, using a CEC differentiation marker, DBA. Different incorporation of deuterium into DNA from each zone of the crypt also verifies sequential cell isolation. DNA from CEC exhibited minimal contamination from fecal bacterial DNA, as confirmed by PCR (data not shown).

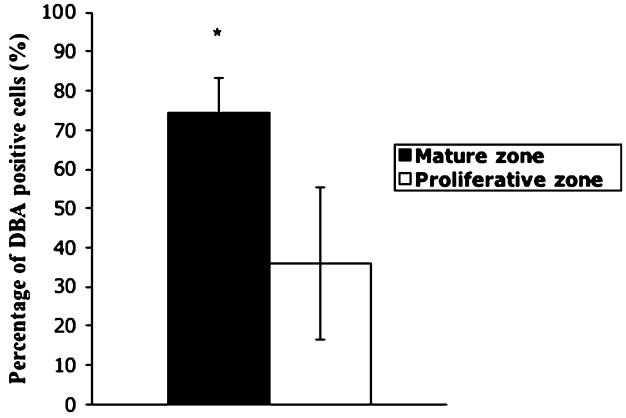

Fig. 3. Confirmation of cell isolation procedure from different zones of the crypt by staining with the CEC differentiation marker, DBA ($n = 3$ each).

Coefficient of variation for measurements of dR enrichments

The coefficient of variation from multiple measurements of dR samples derived from the same CEC sample (same sample divided into 6 aliquots) was small (average ± 0.3%), confirming the reproducibility of the GC/MS method (not shown).

Confirmation of turnover rate by cell cycle analysis

To confirm the CEC turnover rates obtained by the ^2H_2O labeling method, we estimated the turnover time of colonic crypts based on cell cycle analysis (Fig. 4). In the proliferative zone, an average of 16% of cells were in S phase (example shown in Fig. 4). Assuming that the cell cycle in rat colon is approximately 18–22 h [25], it should take 6 times (100%/16%) the cell cycle time or ca. 5–6 days for the proliferative fraction of the colon crypt to be completely turned over. This estimate of turnover time is similar to the results obtained using the ^2H_2O method (6 days, Fig. 2B).

Dose-dependent increases in CEC proliferation induced by CHA in the diet

To test the sensitivity of the new stable isotope method to detect different degrees of CEC proliferation, the known promotional effect of CHA on CECs was exploited [4]. Varying concentrations of CHA were

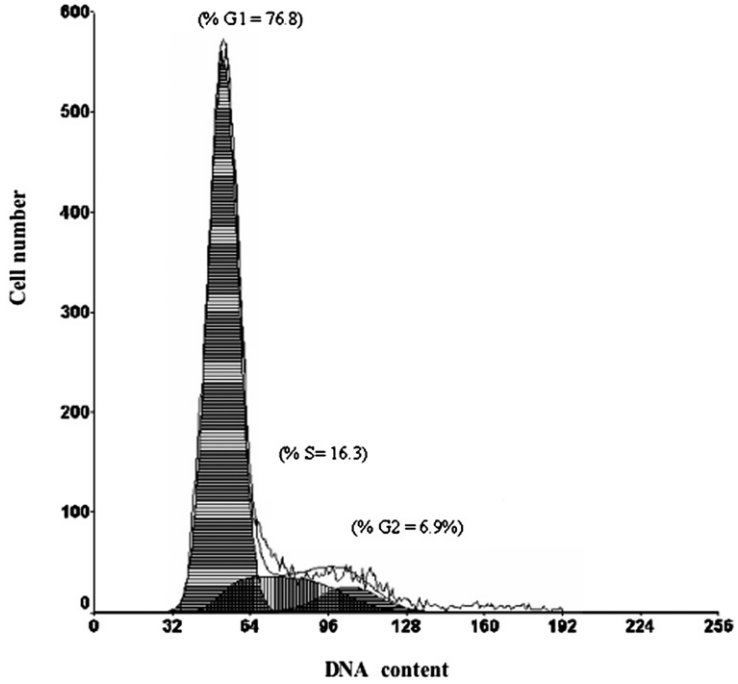

Fig. 4. Confirmation of turnover rate from cell cycle analysis. Cells from F344 rats were stained with propidium iodide. Cells were analyzed by a Beckman-Coulter EPICS XL flow cytometer and the histograms were analyzed by Wyn Cycle (Phoenix Flow Systems, San Diego, CA) software.

added to the diet for 3 weeks and the rats were given ^2H_2O for 3 days before being sacrificed (Fig. 5). In the proliferative zone, the fraction of new cells during 3 days increased progressively from $44 \pm 2\%$ to $52 \pm 8\%$, $60 \pm 12\%$, and $69 \pm 2\%$, with 0%, 0.1%, 0.3%, and 0.5% CHA diets, respectively (mean $\pm$ SD). In the mature zone, CEC proliferation increased from $24 \pm 6\%$ to $47 \pm 10\%$, $49 \pm 6\%$, and $60 \pm 10\%$ with 0%, 0.1%, 0.3%, and 0.5% CHA diets, respectively. Thus, the ^2H_2O labeling method demonstrated an abolition of differences in proliferation rates between mature and proliferative zones of the colon crypts in response to dietary CHA, in addition to the dose-dependent increases in CEC proliferation in each zone.

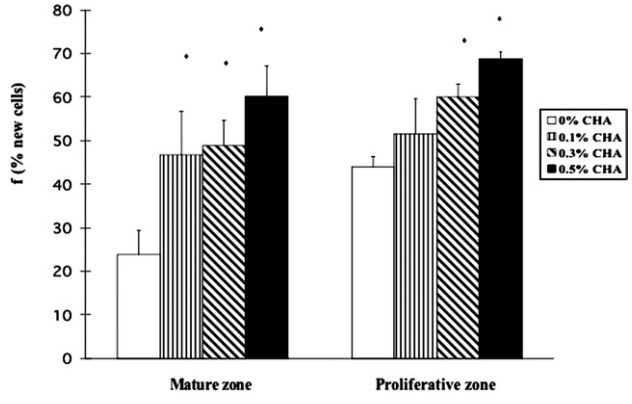

Fig. 5. Measurement of dose-dependent increases in CEC proliferation induced by cholic acid (CHA) in the diet. ^2H_2O labeling was for three days before sacrifice ($n = 4$ for controls, $n = 6$–7 for CHA groups). *, one-way ANOVA ($p < 0.05$, vs. control in each fraction). Values shown are means $\pm$ SD. Note the expansion of the proliferative fraction (i.e., disruption of labeling gradient between mature and proliferative fractions) induced by CHA.

Comparison of the fraction of new cells measured by ^2H_2O and BrdU methods

DNA synthesis rates calculated by the ^2H_2O and BrdU immunohistochemical methods were compared. The correlation (r^2) between the BrdU LI and fraction of new DNA produced from ^2H_2O method was 0.83 ($p < 0.005$, Fig. 6).

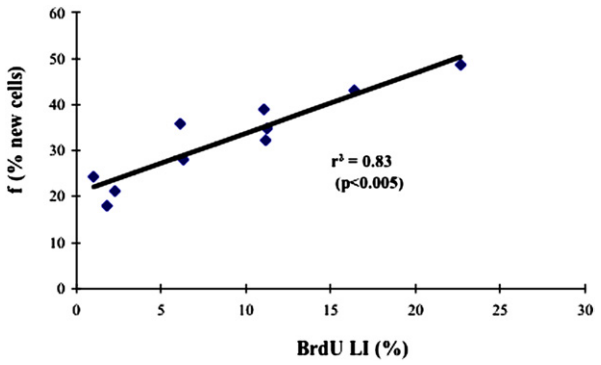

Fig. 6. Comparison of the fraction of new cells measured by ^2H_2O and BrdU labeling index (LI) in the same animal. There was a high correlation ($r^2 = 0.83$, $p < 0.005$) between the two measurements. BrdU LI was measured from immunohistochemically stained tissue samples ($n = 10$).

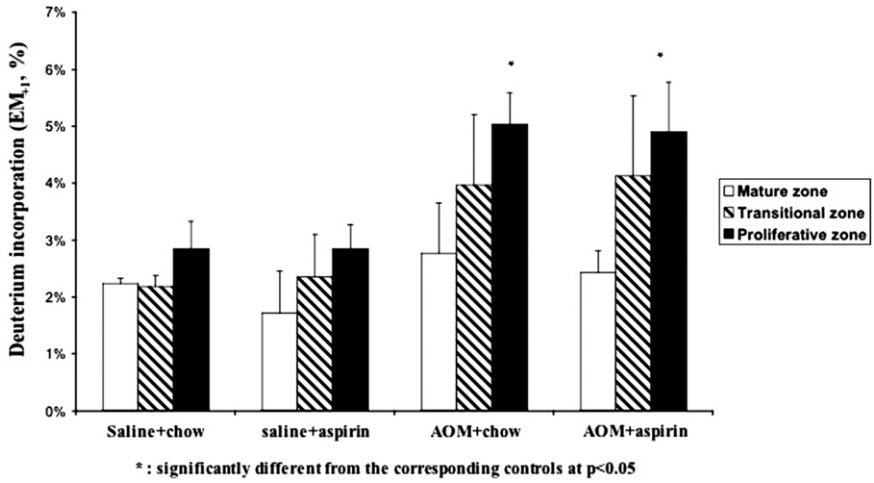

Fig. 7. Increased DNA synthesis with AOM treatment but no changes with aspirin administration. *, one-way ANOVA followed by Fisher's PLSD ($p < 0.05$, vs. each control in each fraction). Values shown are means ± SD. Note the increase in DNA synthesis rates induced by AOM.

AOM treatment increases cell proliferation rates in all fractions of the crypt

With AOM treatment, cell proliferation rates in the proliferative zone of the crypt increased vs. controls (saline + chow and saline + aspirin). AOM + aspirin administration did not change cell proliferation rates compared to the AOM + chow group (Fig. 7).

Discussion

Here, we demonstrate technical as well as practical advantages of the ^2H_2O labeling method [9–11] for measuring DNA synthesis rates and thus CEC proliferation. This method may be useful for the evaluation of novel chemopreventive drugs on colonocyte proliferation in clinical studies in humans, among other potential applications.

The relative ease and reproducibility of the deuterated water technique are worth emphasizing. Body water enrichments have been maintained constant for >70 days in rats [10]. Our findings of a proliferative gradient within the crypt and a physiologically reasonable time lag for progression between proliferative and mature crypt zones support the method (Fig. 2B). Moreover, the small coefficient of variation for multiple measurements of ^2H enrichment in CEC (average ± 0.3%) confirms the analytic reproducibility of the method.

The accuracy of this method was also supported by the similarity between estimates of turnover time by ^2H_2O labeling and by cell cycle analysis results (Figs. 2B and 4), and by the high correlation ($r^2 = 0.83$, $p < 0.005$) between ^2H_2O and BrdU LI results (Fig. 6).

The sensitivity of this method was demonstrated by its ability to detect disruption of the proliferative gradient induced by varying concentrations of dietary CHA, a colon-cancer promoter (Fig. 5). It is important for the method to be sensitive enough to detect different degrees of hyperproliferation since hyperproliferation is typically observed and is believed to be one of the factors responsible for progression to colon tumors [2–6,13]. In experimental animals, many studies have shown that hyperproliferation increases the number of tumors induced by colon carcinogens [3,4,6]. In humans, premalignant lesions of the colon are characterized by increased proliferation rates in, for example, chronic ulcerative colitis and benign adenomas [7,26]. Dysplasia in these premalignant lesions correlates with risk of colon cancer [7,26,27]. Several reports have described progressive increases in proliferative rates from normal mucosa to adenoma and then to carcinoma in humans [2,3,13].

Moreover, the sensitivity of the method for detecting cancer risk was demonstrated by its ability to measure increases in DNA synthesis rates in early carcinogenesis (Fig. 7). These results suggest possible usefulness of the method in detecting risk of colon cancer in preclinical and clinical studies. In addition, aspirin was given for 10 days after AOM in order to study the early effects of aspirin on cell proliferation rates of CEC. Of interest, aspirin administered after AOM did not suppress CEC proliferation rates. Aspirin has been shown to inhibit the formation of aberrant crypt foci (preneoplastic lesions) in rodents that are formed locally and not globally in colon tissue during initiation and post-initiation period [28]. Accordingly, this method can distinguish different mechanisms, (i.e., local vs. global effects and pro- vs. anti-proliferative actions) of drugs in chemoprevention studies. Possibly, aspirin may have acted through other mechanisms such as inducing apoptosis of epithelial [15] and endothelial cells [29], and down-regulation of insulin signaling pathways [16].

In conclusion, we have described a non-toxic, reliable, and a sensitive method using ^2H_2O labeling to measure DNA synthesis rates of CEC and thus proliferation in vivo in normal, promotional, and early carcinogenesis stages. The technique is simple, economical, and can potentially be applied in humans as well as experimental animals. This new method can in principle be applied in humans as a biomarker and for understanding mechanisms of chemopreventive agents against colon cancer.

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