Proliferating Cell Nuclear Antigen: A Marker for Hepatocellular Proliferation in Rodents

by Sandra R. Eldridge,1 Byron E. Butterworth,2 and Thomas L. Goldsworthy2

Two different markers for quantitating cell proliferation were evaluated in livers of control and chemically treated mice and rats. Proliferating cell nuclear antigen (PCNA), an endogenous cell replication marker, and bromodeoxyuridine (BrdU), an exogenously administered DNA precursor label, were detected in formalin-fixed, paraffin-embedded tissues using immunohistochemical techniques. The percentage of cells in S phase (labeling indexes, LI) evaluated as PCNA- or BrdU-positive hepatocellular nuclei was compared in retic tissue sections from animals given BrdU by a single IP injection 2 hr before killing the animals. Ten-week-old male B6C3F1 mice and F344 rats were exposed to known mitogenic hepatocarcinogens, Wy-14,643 (WY) in the diet at 0.1% for 2 days or 1,4-dichlorobenzene (DCB) in corn oil by gavage for 2 days (600 mg/kg/day in mice; 300 mg/kg/day in rats). In mice, PCNA and BrdU hepatocyte LI were similar in control, WY-treated, and DCB-treated animals. In rats, PCNA and BrdU gave similar LI in controls and WY-treated animals. Although PCNA LI was statistically lower than BrdU LI in DCB-treated rats, both PCNA and BrdU LI for DCB-treated rats was increased over LI in control rats. Different patterns of PCNA immunohistochemical staining, interpreted to represent different subpopulations of cells at various phases of the cell cycle, were quantitated using PCNA immunohistochemistry. The proliferating index (PI), defined as the percentage of cells in the cell cycle (G1 + S + G2 + M), was more sensitive than the LI (S phase only) in detecting a chemically induced cell proliferative response. Due to reports of adverse effects of BrdU on cell proliferation, PCNA immunohistochemical methods were used to determine the effect of duration of exogenously administered DNA precursor label (BrdU or [3H]-thymidine [3H-TdR]) on rodent hepatocyte proliferation measurements. PCNA LI were determined in control animals pulse labeled with BrdU or [3H-Tdr, or labeled continuously for 3 or 6 days. PCNA LI did not increase with duration of exposure to BrdU or [3H-Tdr, suggesting that these labeling conditions are not causing a hepatocellular proliferative response. These results demonstrate comparable hepatocyte labeling of cells in S phase in control and chemically treated mice and rats with PCNA and pulse-BrdU labeling methods, supporting the use of PCNA as an alternative marker in either retrospective or prospective cell proliferation studies.

Introduction

An increasing amount of evidence indicates that chemically induced cell proliferation may play a key role in chemical carcinogenesis (1–2). It has been postulated that for nongenotoxic carcinogens, the induction of cell proliferation in the target tissue is an important component of tumor formation (2). Understanding the relationship between chemically induced cell proliferation and carcinogenic activity would be valuable in the investigation of mechanisms of carcinogenesis. Furthermore, this understanding might assist in the development of assays for identifying nongenotoxic carcinogens, the selection of appropriate doses for cancer bioassays, and the improvement of risk assessment models. It has been difficult to formulate a comprehensive database to relate chemically induced cell proliferation to carcinogenesis because quantitative studies in which cell proliferation was measured are lacking.

Traditionally, chemically induced cell proliferation in a target tissue has been quantitated by histoautoradiographic visualization of [3H]-thymidine ([3H]-Tdr) incorporation into the DNA of cells in S phase. More recently, immunohistochemical techniques using a monoclonal

1Pathology Associates, Inc., 4915 D Prospectus Drive, Durham, NC 27713.
2Chemical Industry Institute of Toxicology, P.O. Box 12137, Research Triangle Park, NC 27709.
Address reprint requests to Information Services, CIIT, P.O. Box 12137, Research Triangle Park, NC 27709.
This paper was presented at the Symposium on Cell Proliferation and Chemical Carcinogenesis that was held January 14–16, 1992, in Research Triangle Park, NC.
antibody to 5-bromo-2′-deoxyuridine (BrdU) have been
used to detect cells that have incorporated BrdU
during the S phase of the cell cycle. Both \(^{3}H\)-Tdr and
BrdU are administered exogenously via pulse dosing
or by surgically implanted osmotic pumps to identify
cells in S phase. However, a method to identify replicating
cells using an antibody that recognizes an
endogenous marker of cell replication, proliferating
cell nuclear antigen (PCNA), has been recently
developed (4,5). PCNA is an auxiliary protein of DNA poly-
merase \(\beta\), an enzyme necessary for DNA synthesis (6).
Synthesis of this protein begins in the late G\(_1\) phase of
the cell cycle and peaks during S phase (7). Immunohis-
tochemical detection of PCNA in formalin-fixed,
paraffin-embedded archival rodent tissues has been
achieved using a commercially available antigen
retrieval system based on microwave exposure of his-
tologic sections in the presence of heavy metal salts
(4,8). Furthermore, PCNA analysis has the potential to
identify cells in the different phases of the cell cycle
[i.e., G\(_1\), S, G\(_\text{m}\), and M (5)]. This type of analysis might
reveal potentially critical information on a chemical's
effect on individual cell cycle populations and the
population of proliferating cells. At present, the use and
interpretation of PCNA in cell proliferation studies
requires further validation.

One way to evaluate PCNA as a marker for cell
proliferation is to compare cell labeling indexes (LI, per-
centage of cells in S phase) obtained with PCNA to
BrdU in the same animals. Similar LI have been
obtained with \(^{3}H\)-Tdr and BrdU in control and chemically
treated rodent liver and kidney (9,10). In the experi-
ments presented here, BrdU administered by IP injec-
tion was compared to PCNA for measuring hepato-
cellular proliferation in control and chemically treated
mice and rats to evaluate the feasibility of using PCNA
immunohistochemistry for cell proliferation studies.

Because PCNA is an endogenous marker of replications,
cells, it gives an indication of the S phase portion of
cells at the particular point in time at which the animal
was killed. Thus, using PCNA immunohistochemical
methods to detect cells in S phase is expected to be
analogous to detecting S phase cells using a DNA pre-
cursor label administered by a single pulse injection.
PCNA, however, has the potential to identify the pro-
liferating cell pool, not just cells in S phase. Thus, the
proliferating index (percentage of cells cycling, PI)
obtained with PCNA should be greater than the S
phase LI obtained with PCNA or pulse BrdU. In the
present study, the sensitivity of PCNA to detect a
chemically induced cell proliferative response was
investigated by comparing PCNA PI to PCNA LI.

The pulse labeling method gives an indication of the
level of LI at a specific point in time, whereas continu-
ous labeling via an implanted osmotic pump is cumula-
tive and labels all cells that enter S phase during the
period of infusion of the DNA precursor label. The deci-
sion whether to use pulse or pump label administration
depends on the experimental question and the
turnover rate of the target cell population. Recent
reports have suggested that continuous administration
of BrdU may induce cell proliferation secondary to
cumulative toxicity (11). If BrdU administration affect-
ed the rate of cell proliferation, then the PCNA LI
might be expected to depend on the length of exposure
to BrdU. To further investigate this hypothesis, we
used PCNA analysis on archival material to examine
the effect of duration of exogenously administered
DNA precursor on rodent hepatocyte proliferation
measurements.

Materials and Methods
Tissues
Archival material from a previously conducted cell
proliferation study (10) was used in these experiments.
The experimental design is outlined in Table 1. Briefly,
10-week-old male B6C3F\(_1\), mice and F344 rats were
exposed to either Wy-14,643 (Wy) in the diet at 0.1% for
2, 4 or 5 days, or 1,4-dichlorobenzene (DCB) in corn
oil by gavage for 2, 4, or 5 days (600 mg/kg/day for
mice; 300 mg/kg/day for rats). Controls were included
at each time point.

Five animals per group received either BrdU or \(^{3}H-
Tdr administered by IP injection or continuously for 3
or 6 days by sc-implanted osmotic pumps. For IP injec-
tions, BrdU (100 mg/kg of a 20 mg/mL solution) or \(^{3}H-
Tdr (2 mCi/kg) was administered 2 hr before killing
the animals. Osmotic pumps were implanted in animals
to deliver either BrdU or \(^{3}H\)-Tdr for 3 or 6 days (1
\(\mu\)L/hr for mice, 10 \(\mu\)L/hr for rats). BrdU and \(^{3}H\)-Tdr
were delivered at a rate of 20 \(\mu\)g/hr (65 nmole/hr) and
10 \(\mu\)Ci/hr (1.2 \(\times\) \(10^{-4}\) nmole/hr), respectively, in mice, or
200 \(\mu\)g/hr (650 nmole/hr) and 100 \(\mu\)Ci/hr (12 \(\times\) \(10^{-4}\)
nmole/hr) in rats.

Sections of liver were fixed in 10% neutral-buffered
formalin for 4 days, embedded in paraffin, and stored
for 26 months before performing PCNA immunohis-
tochemistry. Liver sections were cut 4-5 \(\mu\)m thick from
the same tissue blocks that had been previously used
for BrdU immunohistochemistry or \(^{3}H\)-Tdr autoradi-
graphy (10).

| Table 1. Experimental design. |
|-----------------------------|
| Day | 2-hr pulse\(^a\) | 3-day pump\(^b\) | 6-day pump\(^b\) |
|---- |---------------- |---------------- |---------------- |
| -1  | Chemical\(^b\) | Chemical | Implant |
| 0   | Chemical | Chemical/Implant | Chemical |
| 1   | Chemical | Chemical | Chemical |
| 2   | Inject/sacrifice | Chemical | Chemical |
| 3   | Chemical | Chemical | Chemical |
| 4   | Sacrifice | Chemical | Chemical |
| 5   | Sacrifice | | |

\(^a\)Single IP injection of BrdU of \(^{3}H\)-Tdr administered 2 hr before
killing control and treated animals.

\(^b\)Osmotic pumps containing BrdU or \(^{3}H\)-Tdr implanted in control
and treated animals; only controls used in the present study.

\(^c\)"Chemical" denotes WY or DCB. Controls were included at each
time point.
Immunohistochemistry

Previously described methods were followed for PCNA immunohistochemistry (4). Briefly, tissue sections were mounted on 3-aminopropyltriethoxysilane-coated slides to ensure adhesion during processing, deparaffinized in xylene, and passed through graded alcohols. Endogenous peroxidase was inhibited with 3% hydrogen peroxide (8 min). Slides were then immersed in antigen-retrieval solution (BioGenex Laboratories, San Ramon, CA) in a plastic Coplin jar with a loose-fitting screw cap. The Coplin jar was placed in a 720 W microwave oven set on the highest power (10 min for mice and 5 min for rats). Distilled water was added to prevent slide dehydration before reheating in the microwave oven for the same duration. After cooling to room temperature, sections were incubated for 1 hr at room temperature with the primary antibody, monoclonal anti-PCNA 19A2 (Coulter Immunology, Hialeah, FL), at a dilution of 1:400 for 1 hr at room temperature. Subsequently, the slides were incubated for 30 min at room temperature with biotinylated goat anti-mouse IgM (Jackson Immunology Research Laboratory, West Grove, PA) diluted 1:200. Slides were then incubated with streptavidin conjugated to horseradish peroxidase for 30 min at room temperature. The PCNA was localized by a final incubation with the chromagen 3-amino-9-ethylcarbazole (AEC; Zymed Laboratory, Inc., San Francisco, CA). Tissue sections were counterstained with hematoxylin and coverslipped with Crystal mount (Biomedica Corporation, Foster City, CA).

Scoring Labeled Hepatocytes

Positive staining for PCNA was categorized based on cellular distribution and intensity of the red reaction product that correlated with the different phases of the cell cycle as reported by Foley et al. (5). Computer-generated random fields were used to score PCNA by light microscopy. At least 1000 hepatocellular nuclei in the left lobe were counted in at least 6 fields. Typically, 1500-2000 nuclei were counted in PCNA-stained liver sections. Labeling index was calculated by dividing the number of hepatocyte nuclei in S phase by the total number of hepatocyte nuclei counted, and the results were expressed as a percentage. In addition, an index was calculated for G1, G2, or M phase of the cell cycle. A proliferating index was defined as the percentage of cells in the cell cycle, i.e., G1 + S + G2 + M divided by G1 + S + G2 + M + G0. The PCNA LI was compared to the BrdU LI that had been previously quantitated (10).

Statistical Analysis

The Student's t-test for the equality of two means was used to test for significant differences between PCNA and pulse BrdU LI, and LI for control and chemically treated groups. The Student-Newman-Keuls' test for the inequality of unpaired multiple data sets was used to determine significant differences in PCNA LI between pulse-, 3-day, and 6-day pump-labeled animal groups.

Results

Comparison of PCNA to Pulse BrdU

Consistent PCNA staining was achieved in archival liver tissue sections. Staining of PCNA antigen-antibody complexes was categorized based on the cellular distribution and intensity of the reaction product, which was interpreted as representing the different phases of the cell cycle (Fig. 1). Uniform, dark red nuclear staining was judged to be positive for S phase cells. A cell in the G1 phase of the cell cycle had diffuse, stippled nuclear staining that was lighter than a cell in S phase. Cells in the G2 phase exhibited distinct, diffuse cytoplasmic stippling with or without nuclear staining. Mitotic figures were conspicuous with diffuse cytoplasmic stippling.

The percentage of hepatocytes in S phase was generally similar for both PCNA and pulse-labeled BrdU in control and treated mice and rats (Fig. 2). In rats, however, PCNA LI was statistically less than BrdU LI in DCB-treated animals (Fig. 2B). Nevertheless, PCNA LI in DCB-treated rats was significantly greater than in control animals, as observed with BrdU.

Under the treatment regimen and at the time point examined, the distribution of cells in each phase of the cell cycle was altered in WY- and DCB-treated groups as shown in Figure 3. In WY-treated mice, the percentage of hepatocytes in all phases of the cell cycle was increased, whereas in DCB-treated mice only the percentage of hepatocytes in the G1 phase and S phase was slightly increased (Fig. 3A). In WY- and DCB-treated rats, the percentage of hepatocytes in all phases of the cell cycle was increased (Fig. 3B).

The proliferating index as detected by PCNA was a more sensitive indicator of a chemically induced cell proliferative response than LI detected by PCNA (Fig. 4). The fold increase in cell proliferation of treated over control animals was greater for PI than LI.

Effects of Exogenous DNA Precursor Label on Cell Proliferation as Detected by PCNA

Comparable hepatocyte labeling of cells in S phase was obtained with PCNA in control animals that received either BrdU or 3H-Tdr by a 2 hr pulse, a 3-day or 6-day pump (Fig. 5). PCNA LI did not differ between 2 hr pulse-labeled and 3-day or 6-day pump-labeled mice, regardless of the DNA precursor label (Fig. 5A). In rats, the PCNA LI in 2-hr 3H-Tdr-labeled animals was statistically greater than PCNA LI in 3-day and 6-day pump-labeled rats (Fig. 5B). In BrdU-
Figure 1. Immunohistochemical staining for proliferating cell nuclear antigen; G₁, S, G₂, and M phases of the cell cycle identified in rodent liver.

Figure 2. PCNA and bromodeoxyuridine (BrdU) labeling indexes in the liver of control and WY- or dichlorobenzene-treated mice (A) and rats (B). Test chemicals were administered for 2 days. BrdU was administered 2 hr before killing the animals. Liver sections stained for PCNA were from the same tissue blocks used for BrdU immunohistochemistry. Value above each bar represents the mean ± SE of five animals. Percentage of hepatocytes in S phase was determined by counting at least 1000 nuclei as described in Materials and Methods. (*) BrdU and PCNA differ significantly (Student's t-test, p < 0.05).
Figure 3. G₁, S, G₂ and M phase indexes in the liver of control and WY- or dichlorobenzene-treated mice (A) and rats (B) stained by PCNA. Test chemicals were administered for 2 days. Percentage of hepatocytes in each individual phase of the cell cycle was determined as described in Materials and Methods. Value above each bar represents a total of five animals.

Figure 4. PCNA labeling index (LI) and proliferating index (PI) in the liver of control and WY- or dichlorobenzene-treated mice (A) and rats (B). Test chemicals were administered for 2 days. Percentage of hepatocytes in S phase or cycling was determined as described in Materials and Methods. Value above each bar represents the mean ± SE of five animals.
labeled rats, PCNA LI did not differ among 2 hr pulse-, 3-day, or 6-day pump-labeled animals.

**Discussion**

The data presented here support the hypothesis that PCNA may serve as a valuable marker for hepatocyte proliferation. Because PCNA is an endogenous marker of proliferating cells, it can be used in retrospective studies with archival tissues as well as prospective studies. Under the conditions used in these experiments, PCNA was similar to pulse BrdU in detecting both background and chemically induced cell proliferation. The use of PCNA was validated in archival liver tissues, with two species (mouse and rat) and for two chemical carcinogens (WY and DCB) by comparison with previous cell proliferation data gathered via conventional BrdU and H-Tdr techniques. Thus, PCNA would appear to provide a useful means for retrospective assessment of cell proliferation in previously conducted rodent toxicity and carcinogenicity studies in which animals were not administered an exogenous DNA precursor label.

In only one instance was a statistically significant difference in LI between PCNA and pulse BrdU observed. Specifically, in DCB-treated rats, PCNA LI was observed to be less than BrdU LI. However, this difference was slight and was not considered to be biologically significant. Furthermore, PCNA LI for DCB-treated rats was increased over controls, suggesting statistical rather than biological significance.

Not only was PCNA equally as sensitive as pulse BrdU for detecting hepatocytes in S phase, but the PI measured using PCNA was more sensitive than pulselabel methodology or PCNA LI for detecting an induced cell proliferative response. Evidence for this is shown by the fold increase over controls in chemically treated groups, which was greater for PI than for PCNA LI. Furthermore, PCNA detected a similar population of replicating cells as observed by BrdU pump-labeled animals (10). This was evident by the similar pattern of hepatocellular labeling by PCNA and BrdU, which was periportal in WY-treated rats and centrilobular in DCB-treated mice that received 3-day pumps (10). Whereas the pulse-labeling method did not label enough cells to detect a pattern of labeled cell distribution, PCNA immunostaining was able to detect these patterns. This is to be expected because the PI detects cells in the various stages of the cell cycle (G1, S, G2, and M), and not only those in S phase. Thus, the PI would allow for a larger window of observation in which a proliferative effect may be observed and
increase the chance to detect a specific peak of induced proliferation in a target tissue. Furthermore, PI analysis by PCNA has the potential to identify perturbations in the cell cycle, such as a block at the G_1/S phase border, which may lead to an increased understanding of a chemical's effect on cell growth and kinetics.

Detection of different cell subpopulations based on phases of the cell cycle using PCNA methodology may reveal interesting information about the cell cycle, as has been previously speculated. PCNA synthesis begins in G_1 phase and peaks in S phase (7,12). During G_2 with dissolution of the nuclear membrane, PCNA apparently leaks into the cytoplasm. The different staining patterns and intensities were interpreted to represent individual phases of the cell cycle as described by Foley et al. (5). However, further characterization studies are required to confirm this correlation and to clarify the interpretation of such data.

Immunohistochemical detection of PCNA as an alternative method for identifying replicating cells has been compared to autoradiographic procedures with ³H-Tdr and immunohistochemical methods using BrdU or other markers of cell proliferation. These studies have revealed both positive and negative correlations (12–16). Although the reasons for these discrepancies are unknown and warrant further investigation, they may be related to PCNA gene regulation (17), temporal expression of PCNA as a function of the cell cycle in different cell types (12,15), and/or differences between target epitopes of the anti-PCNA antibody used. Finally, cell cycle distribution for markers of cell proliferation differs. For example, BrdU and ³H-Tdr incorporation occurs only during the synthesis of DNA (S phase). PCNA, however, has the potential to identify various stages of the cell cycle (G₁, S, G₂, and M). Therefore, investigators need to specify the characteristics of a positive response. The results presented here indicate that only cells exhibiting distinct nuclear staining for PCNA should be scored as S phase cells to generate a labeling index. Comparisons of labeling index generated using ³H-Tdr or flow cytometric analysis versus PCNA have shown good correlations (13,14).

BrdU pump labeling methods are useful for assessing chronic and/or low levels of chemically induced cell proliferation over a period of time. Recent reports have suggested that continuous administration of BrdU may induce cell proliferation secondary to cumulative toxicity (11). To further investigate this hypothesis, we examined the effect of duration of exogenously administered DNA precursor label on rodent hepatocyte proliferation measurements. To determine whether any effects may be specific for BrdU or generalized for DNA precursor labels, the PCNA LI was examined in the liver of mice and rats following BrdU and ³H-Tdr administration. If BrdU or ³H-Tdr affected cell proliferation, then the PCNA LI would be expected to depend on the length of exposure to BrdU or ³H-Tdr. PCNA LI did not increase with duration of exposure to BrdU or ³H-Tdr, suggesting that under these label-

ing conditions, an increased hepatocyte proliferative response does not occur.

The data presented here further support the use of PCNA immunohistochemical staining in cell proliferation studies. The ability to detect PCNA in archival rodent tissues will permit retrospective cell proliferation studies to be conducted without repeating costly rodent toxicity and carcinogenicity studies. The generation of such data will help clarify the extent to which cell proliferation may be involved in chemically induced carcinogenesis. Analysis of PI and chemically induced cell cycle effects using PCNA immunostaining has the potential to yield novel, important cell proliferation data and warrants further investigation.

We thank Otis Lyght for immunohistochemical staining and Julie Foley and Robert Maronpot for helpful discussions.

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