Detection and Quantification of Ploidy, Nuclearity, and DNA Synthesis in Rat Hepatocytes after Administration of a Peroxisome Proliferator

by J. A Styles

The peroxisome proliferator methylclofenapate (MCP) induces species-specific liver growth and cancer in rats and mice. The acute hyperplastic effects of MCP were studied in rats given MCP (25 mg/kg by daily gavage) and injected IP (0.5 mL, 50 mM) with bromodeoxyuridine (BrdU) 6 hr before each sampling time. The animals were killed at 6-hr intervals and hepatocyte suspensions were prepared from their livers by collagenase perfusion. The cells were stained with propidium iodide (PI) and BrdU antibody combined with fluorescein isothiocyanate (FITC). DNA content (PI-red fluorescence) and S phase (BrdU/FITC-green fluorescence) were analyzed simultaneously by two-parameter flow cytometry and the frequency of S phase in different ploidy classes determined. At the same time, S-phase cells from different ploidy groups were sorted onto slides by fluorescence-activated cell sorting and examined microscopically to determine the frequency of binucleated cells undergoing DNA synthesis. The results show that MCP-induced acute hyperplasia occurs mainly in a sensitive subpopulation of binucleated hepatocytes.

Introduction

In most investigations of cell proliferation in vivo, the population under study consists of mononuclear diploid cells that undergo replication via normal, complete division cycles. Because the principal phenomena associated with the cell cycle (DNA replication and mitosis) are sequential, only one is normally measured, and it is usually adequate to quantify proliferative activity by measuring the frequency of cells either in S phase by autoradiography or by BrdU-antibody immunohistochemistry (the labeling index); or the frequency of cells undergoing mitosis (the mitotic index) after injection of colchicine to arrest cells in metaphase.

The mammalian liver normally exhibits near-quotient proliferative activity, but it is capable of very rapid regenerative cell division after toxic or physical injury (1). In rats and mice, the liver also undergoes non-regenerative hyperplasia after the administration of peroxisome proliferators (2).

The livers of rats and mice, unlike those of other mammals, consist mainly of hepatocytes that contain two classes of cells with respect to nuclei and several ploidy classes. These classes of hepatocytes arise as the result of modified cell-division cycles (3–5). The peculiar cytological composition of the rodent liver has, until recently, caused difficulties in measuring cellular ploidy and cell proliferation and interpreting cell-cycle observations. The presence of a karyotypically nonhomogeneous hepatocyte population has limited the accuracy with which DNA content and nuclearity can be estimated in histological sections. Furthermore, the use of autoradiography for the quantification of S-phase synthesis in histological sections of liver has been complicated not only by the problem of variable ploidy and nuclearity, but also by the near impossibility of discerning morphological cellular detail obscured by silver grains. The measurement of mitotic activity in the rodent liver is also complicated by its peculiar cytology. Here the problems encountered are similar to those for S-phase activity; the ploidy and nuclearity of sectioned mitotic figures cannot be assessed accurately.
Materials and Methods

Chemicals. Pure methylclofenapate (MCP, no impurities detected by standard analytical techniques) was obtained from Lancaster Synthesis (9). Bromodeoxyuridine (BrdU) was supplied by Sigma. Propidium iodide was purchased from Polysciences. Purified anti-BrdU antibody conjugated with fluorescein isothiocyanate (FITC) was obtained from Becton-Dickinson. Corn oil was supplied by Kraft foods.

Animals. Male Alpk:AP strain rats, 200-250 g weight were used. Environmental conditions were as described previously (2). After treatment, the animals were killed by deep anesthesia followed by exsanguination by perfusion in groups of three for each time and dose group.

Dosing. MCP or corn oil were administered by gavage in a dosing volume of 0.5 mL/100 g body weight. MCP was made up at a concentration of 5 mg/mL in corn oil and administered at a dose of 25 mg/kg body weight (2).

Labeling of Cells in DNA Synthesis with BrdU. BrdU was administered to all animals by IP injection (0.5 mL of 50 mM solution in water) 6 hr before killing.

Isolation and Preparation of Hepatocytes. The procedure for isolation and preparation of hepatocytes was as described previously (2,6-8). After collagenase perfusion, the lobes of the liver were excised carefully from each animal and placed in 30 mL of ice-cold PBS-EDTA. The lobes were minced with scissors and the crude cell suspension filtered through a fine mesh gauze and centrifuged for 2 min at 20g. The supernatant was removed by aspiration, the cells suspended carefully in 30 mL of PBS-EDTA, and the centrifugation and suspension was repeated twice. After the final centrifugation, the cell pellet was reduced to about 10 cm³ if necessary and fixed. The hepatocytes were fixed by the dropwise addition of 20 mL of 70% ethanol at -20°C while the pellet was slowly agitated on a vortex. The cells were left overnight in a refrigerator at 4°C. The fixed cells were further prepared for flow cytometric analysis (2,6). Cells in S phase were stained with BrdU-FITC, and all cell nuclei were stained with PI.

Analysis of Hepatocytes for DNA Content, S Phase, and Nuclearity by Flow Cytometry and Microscopy. Cells were analyzed by two-parameter flow cytometry using an Ortho 2150 Cytofluorograf. Hepatocytes were illuminated by an argon laser at 200 mW and a wavelength of 488 nm: DNA content was detected by PI fluorescence (red), and the presence of BrdU monoclonal antibody was detected by FITC fluorescence (green). Cells in S phase were sorted by fluorescence-activated cell-sorting onto microscope slides using the regions shown in Figure 1. The cells were examined microscopically to determine their nuclearity (2,6).

Results

S Phase Response in Hepatocytes. The incidence of S phase in hepatocytes at time intervals after administration of MCP is given in Figure 1. These results show that the hyperplasia elicited by MCP appears to be an acute, transient phenomenon.

Nuclearity Analysis. Figure 2 shows the frequency of 2 x 2N hepatocytes. At the outset of dosing the hepatocyte population consisted of approximately 10% 2N, 23% 2 x 2N, and 67% 4N cells. After the administration of 25 mg/kg MCP, there was a decrease in the proportion of 2 x 2N cells to about 9% and an increase in 4N cells to about 81% (data not shown), while the fraction of 2N cells remained unchanged.

S Phase Response in Nuclear Classes. Figure 3 shows the frequency of 2 x 2N hepatocytes in S phase as a percentage of the total hepatocyte population. There were a few 4N hepatocytes containing label (data not shown), and these were possibly derived from 2 x 2N cells.

Discussion

Flow cytometry and fluorescence-activated cell sorting, used in conjunction with quantitative fluorescent stains for DNA and fluorescently labeled antibodies to BrdU (9-12), have permitted the rapid and precise quantification of cell proliferative activity in the rodent liver. Studies using these techniques have revealed that proliferative activity of hepatocytes may occur in different subpopulations of cells depending on the kind of toxicological injury inflicted on the animal (2). The chief deficiency in the use of flow cytometry is the unavoidable loss of tissue architecture and the concomitant loss of information with respect to the anatomical distribution of cell proliferative activity. This problem may partly be overcome by combining flow cytometry of hepatocyte suspensions obtained by collagenase perfusion with light microscopy of sections of liver in which S phase cells are labelled with BrdU and visualized by immunohistochemical stains.
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REFERENCES

1. Michalopoulos, G. Liver regeneration: molecular mechanisms of growth control. FASEB J. 4: 176–187 (1990).
2. Styles, J. A., Kelly, M., Pritchard, N. R., and Elcombe, C. R. A species comparison of acute hyperplasia induced by the peroxisome proliferator methylofenapate: involvement of the binucleated hepatocyte. Carcinogenesis 9: 1647–1655 (1988).
3. Bohm, N., and Noltemeyer, N. Development of binuclearity and DNA polyploidization in the growing mouse liver. Histochemistry 72: 55–66 (1981).
4. Brodsky, W. Y., and Uryvaeva, I. V. Cell polyploidy: its relation to tissue growth and function. Int. Rev. Cytol. 50: 275–332 (1977).
5. Sattler, C. A., Sawada, N., Sattler, G., and Pitot, H. C. Electron microscopic and time lapse studies of mitosis in cultured rat hepatocytes. Hepatology 8: 1540–1549 (1988).
6. Styles, J. A., Kelly, M., and Elcombe, C. R. A cytological comparison between regeneration, hyperplasia and neoplasia in the rat liver. Carcinogenesis 8: 391–399 (1987).
7. Berry, M. N., and Friend, D. S. High-yield preparation of isolated rat liver parenchymal cells. J. Cell Biol. 43: 506–520 (1969).
8. Seglen, P. O. Preparation of isolated rat liver cells. In: Methods in Cell Biology, Vol. 13, Academic Press, New York, 1976, pp. 29–83.
9. Lanier, T. L., Berger, E. K., and Eacho, P. I. Comparison of 5-bromo-2-deoxyuridine and [3H]thymidine for studies of hepatocellular proliferation in rodents. Carcinogenesis 10: 1341–1343 (1989).
10. Goldsworthy, T. L., Morgan, K. T., Popp, J. A., and Butterworth, B. E. Guidelines for measuring chemically induced cell proliferation in specific rodent target organs. In: Chemically Induced Cell Proliferation: Implications for Risk Assessment (B. E. Butterworth, Ed.), Wiley-Liss, 1991, pp. 253–284.
11. Eldridge, S. R., Tilbury, L. F., Goldsworthy, T. L., and Butterworth, B. E. Measurement of chemically induced cell proliferation in rodent liver and kidney: a comparison of 5-bromo-2'-deoxyuridine and [3H]thymidine administered by injection or osmotic pump. Carcinogenesis 11: 2245–2251 (1990).
12. Smith, P. F., O'Brien, K. A., and Keenan, K. P. Evaluation of bromodeoxyuridine labeling in hepatomegaly produced by peroxisome proliferation or P450 induction in rodents. In: Chemically Induced Cell Proliferation: Implications for Risk Assessment (B. E. Butterworth, Ed.), Wiley-Liss, 1991, pp. 285–289.