Measurement of Ploidy and Cell Proliferation in the Rodent Liver

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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 phenomena associated with the cell cycle are sequential, only one is normally measured and it is usually adequate to quantify the proliferative activity in one of two ways. The first involves labeling the cells undergoing semi-conservative DNA synthesis with a radioactive DNA precursor, preparing autoradiographs of histological sections, and counting labeled nuclei. The other commonly studied parameter of cell proliferation is mitotic activity. The livers of rats and mice, unlike those of other mammals, consist mainly of hepatocytes that contain two classes of cell with respect to nuclei and several ploidy classes. These classes of hepatocytes arise as the result of modified cell division cycles. The peculiar cytological composition of the rodent liver has, until recently, caused difficulties in the measurement and interpretation of cell ploidy and cell proliferation by the above methods. Flow cytometry and fluorescence-activated cell sorting used in conjunction with quantitative fluorescent stains for DNA and fluorescently labeled antibodies to bromodeoxyuridine 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.

Introduction

Cell division is a fundamental biological phenomenon that has been studied in many organisms and tissues. For most adult tissues in which cell renewal occurs, the cell cycle is a predictable process and normally involves complete cycles in a fraction of the cell population. These cycles involve diploid cells, each of which replicates its genome and then segregates the resulting daughter chromosomes between diploid daughter cells, replacing cells that have been lost due to death or ablation. Cell loss and renewal in an adult organ or tissue takes place in a controlled manner, with the rate of cell renewal in an unperturbed organ having a characteristic value. Thus, in adult tissues the size of a cell population is maintained at a constant level by a cell flux, and any change in the components of the flux (rate of cell division, lifespan of cells, or rate of death) will alter the size of the population. Of these parameters, the easiest to study and quantify is cell division, although the process of cell death (including programmed cell death or apoptosis) has increasingly occupied the attention of investigators (1,2). In toxicology, changes in cell division cycle kinetics elicited by xenobiotics, resulting either in degeneration, regeneration, or hyperplasia, have long been considered to be important events in the etiology of pathological conditions.

In eukaryotic organisms, cell division is under the control of an array of genes that are collectively referred as the cell division cycle (cdc) genes. These have been most extensively studied in yeasts and other lower eukaryotes, but mammalian homologues have been described that, not surprisingly, exhibit a considerable degree of conservation (3-6). Furthermore, evidence is emerging that some cdc genes in mammals are homologous to proto-oncogenes (7-9).

Cell Division and the Development of Polyploidy in the Rodent Liver

After birth, the mammalian liver undergoes a considerable increase in size by normal cell division of all the cell types present. In rodents, these normal division cycles soon undergo a change in control in the
parenchymal cells such that, after about 2 weeks of age, binucleated hepatocytes arise (10,11). The formation of binucleated cells appears to be due to the suppression of cytokinesis, the final phase of the division cycle, rather than to cell fusion, because labeling experiments have revealed the presence of label in binucleates (11–14). Shortly after the appearance of binucleated cells, polytoid (tetraploid) mononucleated cells are formed. The mechanism for the genesis of mononucleated tetraploid hepatocytes is not completely clear but appears to occur via binucleated cells: some in vitro studies suggest that S phase occurs simultaneously in both the nuclei, which then enter mitosis when the two separate spindles fuse and a single metaphase plate forms. Anaphase, telophase, and cytokinesis follow, resulting in the formation of two mononucleated tetraploid daughter cells (15). Alternatively, it has been suggested from time-lapse and electron microscopic studies that the two nuclei undergo S phase simultaneously and then enter mitosis, in which a single spindle is formed, and the subsequent chromosomal segregation and cytokinesis are normal, with the subsequent production of two tetraploid cells (16).

In the guinea pig, the process of polyploidization is limited to the production of a small fraction of binucleated hepatocytes (17), and in the hamster a small population of tetraploid cells also appears (17), but in rats and mice polyploidization affects the majority of hepatocytes and is a progressive phenomenon that continues throughout the life of the animal (11–14). In rats the rate of polyploidization slows markedly by the age of about 10 weeks and the liver consists mainly of tetraploid cells (2 × 2n and 4n) and some octoploids (2 × 4n and 8n), but in the mouse the phenomenon progresses to a greater extent, and higher multiples of the diploid DNA content may be observed as the animal ages. The proportion of binucleated hepatocytes is characteristic of a given rodent species and remains fairly constant throughout life (11,12). This indicates that some binucleated cells are intermediates between ploidy states and may undergo a further modified division cycle to produce the succeeding higher ploidy level.

Although the mechanism by which binucleation and polyploidization occur in rodent hepatocytes has been partly elucidated, there is yet no explanation as to its significance. The behavior of the rodent liver in response to various types of injury suggests that is influenced by the ploidy conditions of the hepatocytes.

Cell Proliferation in Rodent Liver

In the adult mammalian liver, there is almost no cell proliferative activity. In comparison with tissues such as the bone marrow, intestinal epithelium, or the skin, where mitotic activity may easily be observed in sectioned material, the appearance of a mitotic figure in a liver section is extremely rare, even after administration of a mitotic blocking agent such as colchicine.

After mechanical or toxic injury, however, the liver undergoes rapid and extensive proliferation until the weight of the organ is restored to normal, at which point the rate of cell division subsides to its normal, near-quiescent levels.

DNA-reactive hepatocarcinogens induce changes in the proportions of the different ploidy and nuclearity classes in the livers of rats and mice (19–28). The carcinogens appear to provide cell division initially in rat and mouse binucleated hepatocytes, which undergo cytokinesis, thus completing the cell cycle and reducing the proportion of binucleated cells (29). Subsequent to this stage, some diploid cells acquire constitutive cycling capacity and give rise to morphologically and biochemically altered nuclei that consist mainly of diploid cells (26). It is not known whether any of these nuclei originate from the binucleated cells that divided, but the change in control of the division cycle in these cells probably involves alterations in expression of edc genes.

There are chemicals that stimulate cell division in the hepatocyte population of rodents at non-neurogenic doses—such as 4-acetylamino-fluorene, appear to be mitogenic but non-carcinogenic (30), while a large and structurally diverse group of chemicals induces liver growth and cancer (31). The compounds in the latter group are non-DNA reactive (32–40), and they elicit liver growth and cancer that is rodent specific (31). The liver growth phenomena that are induced are hepatocyte hypertrophy (smooth endoplasmic reticulum prolifération and usually peroxisome proliferation) and hyperplasia (41–46). Both these phenomena are induced acutely by peroxisome proliferators, but with continued administration of the chemicals only the increased levels of peroxisomes are maintained (31). The hyperplasia appears to occur principally in a fraction of the binucleated hepatocyte population (47–51). Unlike the response elicited by genotoxic hepatocarcinogens, where the cytokinesis is not preceded by S phase and the cells simply complete their interrupted cycle, the liver growth-inducing agents elicit S phase in the binucleates, which then proceed via the normal route for polyploidization, each producing two daughter mononucleated tetraploid cells.

Measurement of Cell Proliferation in Rodent Liver

In most organs in which cell proliferation occurs, the cell cycle proceeds through a series of predictable stages, and each progenitor diploid cell gives rise to two diploid daughter cells per cycle. The occurrence of cell division is usually detected and quantified in one of two ways: by detecting DNA replication during S phase and by observing mitosis.

The occurrence of S phase has, until recently, been studied through the use of radiolabeled precursors, usually tritiated thymidine (for autoradiography of sectioned material or cell cultures). The advent of bromodeoxyuridine (BrdU) labeling and its detection by
monoclonal antibodies has permitted the study of S phase by nonradiolabeling techniques in tissue sections, cultured cells, and in cell suspensions by flow cytometry.

The duration of the G1 and G2 phases of the cell cycle varies within and between cell types (52,53); S phase typically lasts 6-12 hr. and mitosis is usually complete in less than 1 hr. In any method used to detect the occurrence of S phase, the sensitivity of the assay is increased directly in proportion to the persistence of the label. Because all the DNA precursors have a short biological half-life in vivo, maintenance of adequate tissue levels has commonly been achieved by repeated injection or administration in drinking water. Owing to the rate at which precursors are excreted, neither of these methods is entirely satisfactory because the tissue levels of labels fluctuate. Long periods of labeling at constant tissue levels may be achieved through the use of osmotic minipumps (43,54). Studies in rodent liver in which radiolabeling and BrdU labeling by minipump were compared showed quantitatively similar results (55). Methods to quantify the rate and duration of S phase require pulse labeling methods to avoid labeling S phase in daughter cells.

Mitosis (M phase), as an index of cell proliferative activity, is intrinsically less sensitive than S phase because it is of much shorter duration and hence the fraction of the cycling population in M phase is smaller than that in S phase. The detection of mitotic activity, as distinct from observation of the stages of mitosis, has been achieved by extending the period of metaphase. This is most commonly done by arresting cells in metaphase using the spindle poison colchicine. The use of colchicine blockade is limited to a few hours duration and is therefore less sensitive than S phase labeling for cell proliferation. It has the advantage of simplicity because it requires no additional preparative stages to the usual histological procedures. Mitotic cells may also be detected by flow cytometry in conjunction with specific antibodies, providing the cell type under investigation can withstand isolation (55).

Cell proliferative activity in the rodent liver may be detected in the same way as in other organs and tissues. The measurement of proliferation is problematic because the liver parenchymal cell population is not homogeneous, consisting of mono- and binucleated cells and multiples of the diploid component of DNA. As described above, these subpopulations of cells arise as the result of arrested or altered division cycles, and the occurrence of S phase and mitosis cannot be quantified and interpreted in conventional terms.

Studies on hepatocyte proliferation elicited in response to various types of liver injury in rodents have revealed that these involve different subpopulations of hepatocytes. To elucidate which subpopulations of hepatocytes are undergoing cell proliferation, the most precise method requires the use of flow cytometry, fluorescence-activated cell sorting, and conventional microscopy (18,47). Hepatocytes in S phase are labeled in vivo with BrdU, the livers perfused with collagenase, and the resulting cell suspension separated by low-speed centrifugation (56-57). The hepatocyte fraction is fixed and the DNA stained with propidium iodide (PI). Cells in S phase, containing BrdU, are reacted with BrdU antibody and stained with fluorescein isothiocyanate (FITC). Both these fluorochromes are excited by an argon-ion laser beam tuned to emit at a wavelength of 488 nm: the PI emits red fluorescence and the FITC emits green. The cell suspension is analyzed and sorted simultaneously using two-fluorescence flow cytometry. This method allows the ploidy of the cells and the incidence of cells in S phase in different ploidy groups to be determined. The cells are sorted onto slides and examined microscopically using fluorescence optics to determine the proportion of mono- and binucleated cells in S phase in comparison to the total fraction of binucleated cells.

The chief deficiency in the use of flow cytometry is due to the obligatory use of cell suspensions and the resulting loss of all information relating to the anatomical location of S phase activity. This deficiency may, at least in part, be remedied by combining the use of flow cytometry on hepatocyte suspensions with light microscopic examination of liver sections in which the BrdU in S phase cells is visualized by immunohistochemical stains.

REFERENCES

1. Arends, M. J., and Wyllie, A. H. Apoptosis: mechanisms and role in pathology. Int. Rev. Exp. Pathol. 32: 223-254 (1991).
2. Schulte-Hermann, R., Timmermann-Trosiener, I., Barthel, G., and Bursch, W. DNA synthesis, apoptosis, and phenotypic expression as determinants of growth of altered foci in rat liver during phenobarbital promotion. Cancer Res., 50: 5127-5135 (1990).
3. Hayles, J., and Nurse, P. A review of mitosis in the fission yeast Schizosaccharomyces pombe. Exp. Cell Res. 184: 273-286 (1989).
4. Cross, F., Roberts, J., and Weintraub, H. Simple and complex cell cycles. Annu. Rev. Cell Biol. 5: 941-986 (1991).
5. Nurse, P. Cell cycle control genes in yeast. Trends Genet. 1: 51-55 (1985).
6. Nugent, J. H. A., Alfa, C. E., Young, T., and Hyams, J. S. Conserved structural motifs in cyclins identified by sequence analysis. J. Cell Sci. 99: 669-674 (1991).
7. Ron, D., Zannini, M., Lewis, M., Wickner, R. B., Huh, L. T., Graziani, G., Tronick, S. R., Aaronson, S. A., and Eva, A. Region of proto-dbl essential for its transforming activity shows sequence similarity to a yeast cell-cycle gene CDC24 and the human breakpoint cluster gene ber. New Biol. 3: 372-379 (1991).
8. Marshak, D. R., Vandenberg, M. T., Bae, Y.S., and Yu, I. J. Characterization of synthetic peptide substrates for p34cdc2. J. Cell Biochem. 45: 391-400 (1991).
9. Calabretta, B., Kaczmarek, L., Mars, W., Ochoa, D., Gibson, C. W., and Hirschhorn, R. Cell-cycle specific genes differentially expressed in human leukaemias. Proc. Natl. Acad. Sci. U.S.A. 82: 4463-4467 (1985).
10. Nadal, C., and Zajdela, F. Polyploidy somatique dans le foie de rat. I. Le rôle des cellules binucléées dans la genese des cellules polyploïdes. Exp. Cell Res. 42: 99-115 (1966).
11. Bohm, N., and Nolemeyer, N. Development of binuclearity and DNA polyploidy in the growing mouse liver. Histochemistry 72: 55-66 (1981).
12. Severin, E., Willers, R., and Bettecken, T. Flow cytometric analysis of mouse hepatocyte ploidy II. The development of polyploid patterns in four mouse strains with different life spans. Cell Tissue Res. 229: 493–502 (1984).

13. Brodsky, W. Y. and Urry, E. V. Cell polyploidy: its relation to tissue growth and function. Int. Rev. Cytol. 50: 275–233 (1977).

14. Albert, M., and Geschwind, I. I. The development of polymatous in rat liver. Exp. Cell Res., 15, 230–232 (1958).

15. Carrieri, R. The growth of liver parenchymal nuclei and its endocrine regulation. Int. Rev. Cytol. 25: 201–277 (1969).

16. James, J. R. The genesis of polyploidy in rat liver parenchymal cells. Eur. J. Cell Biol. 15: 410–417 (1977).

17. 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: 1450–1459 (1988).

18. Styles, J. A., Kelly, M., Pritchard, N. R., and Elcombe, C. R. A species comparison of acute hyperplasia induced by the peroxi-

19. Barton, A. D. Cell proliferation in precancerous liver: relation to presence and dose of carcinogens. J. Natl. Cancer Inst. 25: 1215–1255 (1959).

20. Simard, A., Cousineau, G., and Daoust, R. Variations in the cell cycle during azo dye hepatocarcinogenesis. J. Natl. Cancer Inst. 41: 1257–1263 (1968).

21. Neal, G. E., Judah, J. J., and Butler, W. H. Some effects of acute and chronic dosing with aflatoxin B1 on rat liver nuclei. Cancer Res. 36: 1771–1778 (1976).

22. Irving, C. C., Roszell, J. A., and Freli, J. L. Effects of chronic feeding of 2-acetylaminofluorenone on nuclear populations in rat liver. Adv. Enzyme Regul. 16: 306–377 (1977).

23. Schwartz, B. E., Petterson, E. O., Shabib, C., and Seglen, P. O. Emergence of a population of small, diploid hepatocytes during hepatocarcinogenesis. Carcinogenesis. 5: 1267–1275 (1984).

24. Seglen, P. O., Schwartz, B. E., and Saeter, G. Changes in cellular ploidy and autophagic responsiveness during rat liver carcinogenesis. Toxicol. Pathol. 14: 342–348 (1986).

25. Deleener, A., Castelain, P., Freut, V., and Gerlache, J., Alexandre, H., and Krishn, V. M. Changes in nuclear transcriptional activity and nuclear DNA content during the first steps of rat hepatocarcinogenesis. Carcinogenesis. 5: 195–201 (1987).

26. Styles, J. A., Elliott, B. M., Lefevre, P. A., Robinson, M., Pritchard, N. R., Hart, D., and Ashby, J. Irreversible depression in the ratio of diploid liver nuclei in rats treated with 3'-methyl-4-dimethyl aminoazobenzene (3'-M). Carcinogenesis. 6: 21–28 (1985).

27. Kelly, M. D., Styles, J. A., and Pritchard, N. R. Analysis of cytological changes in hepatocytes from rats dosed with 3'-methyl-4-dimethyl aminoazobenzene: initial response appears to involve cytokinesis of binucleated cells. Cancer Lett. 38: 1–4 (1990).

28. Ashby, J., Lefevre, P. A., Burilson, B., and Beije, P. Potent mitogenic activity of 4-acetylaminofluorenone to the rat liver. Mutation Res. 172: 271–279 (1986).

29. ECETOC. Hepatic Peroxisome Proliferation, Monograph No. 17. European Chemical Industry Ecotoxicology and Toxicology Centre, Brussels, 1991.

30. Elliott, B. M., and Elcombe, C. R. Lack of DNA damage or lipid peroxidation measured in vivo in the rat following treatment with peroxisome proliferators. Carcinogenesis 8: 1213–1218 (1987).

31. Butterworth, B. E., Bermudez, E., Smith-Oliver, T., Earle, L., Cattley, R., Martin, J., Popp, J. A., Strom, S., Jirtle, R., and Michalopoulos, G. Lack of genotoxic activity of di-2-ethylhexylphthalate in rat and human hepatocytes. Carcinogenesis 8: 1329–1336 (1984).

32. Cattley, R. C., Smith-Oliver, T., Butterworth, B. E., and Popp, J. A. Failure of the peroxisome proliferator WY-14,643 to induce undulated DNA synthesis in rat hepatocytes following in vivo treatment. Carcinogenesis 9: 1179–1183 (1988).

33. Cattley, R. C., Richardson, K. K., Smith-Oliver, T., Popp, J. A., and Butterworth, B. E. Effect of peroxisome proliferator carcino-

34. Conway, J. G., Tassetowski, K. E., Olson, M. J., Cattley, R. C., Marsman, D. S., and Popp, J. A. Relationship of oxidative damage to the hepatocarcinogenicity of the peroxisome proliferators di-(2-ethylhexyl)phthalate and WY-14,643. Carcinogenesis 10: 515–520 (1989).

35. Berr, F., Staub, W., Kelly, S., Wazecth, F., and Bentley, P. Assessment of peroxisome proliferation and liver growth stimula-

36. Styles, J. A., Bybee, A., Pritchard, N. R., and Kelly, M. D.
Studies on the hyperplastic responsiveness of binucleated rat hepatocytes. Carcinogenesis 11: 1149–1152 (1990).

51. Styles, J. A., Kelly, M., Elcombe, C. R., Bybee, A., and Pritchard, N. R. Recovery of hyperplastic responsiveness in rat liver after dosing with the peroxisome proliferator methylclofenapate. Carcinogenesis 12: 2127–2133 (1991).

52. Brooks, R. F. The transition probability model: successes, limitations and deficiencies. In: Temporal Order (L. Rensing and N. I. Jager, Eds.) Springer-Verlag, Berlin, 1985.

53. Shields, R. Transition probability and the origin of variation in the cell cycle. Nature 267: 704–707 (1977).

54. 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).

55. Clevenger, C. V., Epstein, A. L., and Bauer, K. D. Quantitative analysis of a nuclear antigen in interphase and mitotic cells. Cytometry 8: 280–286 (1987).

56. Seglen, P. O. Preparation of isolated rat liver cells. Meth. Cell Biol. 13: 29–83 (1976).

57. Berry, M. N., and Friend, D. S. Higher yield preparation of isolated rat liver parenchymal cells. J. Cell Biol. 43: 506–520 (1969).