Hepatic Nuclear Ploidy Distribution of Dietary-Restricted Mice

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Hepatic parenchymal cells in most adult mammals are polyploid, with most of the cells in the quiescent or low-proliferation state. Polyploidization has been related to carcinogenesis and aging, and both endpoints are significantly affected by dietary restriction (DR). Direct measures of hepatic nuclear polyploidization in DR B6C3F1 mice have not been examined. We examined the effect of DR on distributions of nuclear ploidy in both sexes and on different age groups of B6C3F1 mice. Differences between young and old male mice and between old male and female mice were also compared. Hepatic nuclear ploidy values were measured by flow cytometry. The DNA histograms were analyzed for the percentage of nuclei having different classes of DNA content by gating channels between the areas under the peaks of diploid, tetraploid, and octaploid. The results indicate that 1 or 26 months of DR started at 4 months of age did not alter hepatic nuclear ploidy distributions in young and old mice. Our data suggest that in the male mouse, polyploidization is established by 5 months of age for hepatic nuclei and that ploidy classes are affected by sex at 30 months of age. For females, effects in the octaploid nuclei are seen as a result of DR.

Introduction

It has been reported that liver parenchymal cells in most adult mammals are polyploid (1–8). Hepatic parenchyma consists of a population of mononuclear and binuclear cells of different ploidy classes. Polyploidization of mammalian hepatocytes is regarded as an equivalent of cell multiplication (9). In adult mice, about 65–85% of liver cells are polyploid (1,6,10). Polyploidization has been reported to be related to the aging process and carcinogenesis (11–16), with the degree of hepatic polyploidy being age dependent (1,3,5–17).

Dietary restriction (DR) is an effective means of extending maximum life span, retarding the onset of senescence, and lowering incidences of many tumors in rodents (18–21). DR delays the time of occurrence of various age-associated diseases, including cancer (22–24), and is a useful modulator of chemical toxicity. However, its mode of action remains to be elucidated. DR may inhibit cell division in specific tissues in rats (25–26) and mice (27) and, perhaps, inhibits the growth of normal cells and/or cell variants that have the capacity to become neoplastic.

Cell proliferation can be determined by cell-cycle analysis using flow cytometry based on the measurement of DNA content (28–29) and the incorporation of a DNA precursor label such as bromodeoxyuridine [BrdU (30)]. The data obtained from analysis of DNA distributions in each phase of the cell cycle provides information about the proliferative activity (31).

The effect of the DR on liver polyploidy has been reported by studying the diameter or volume of the hepatic nuclei (8,32). However, hepatic nuclear ploidy values obtained by direct measurement of the nuclear DNA content have not been examined in DR mice. We describe here a method of measuring hepatic nuclear ploidy classes in DR mice, the effect of DR on hepatic nuclear ploidy classes in mice, and the effect of DR on hepatic nuclear ploidy distributions in 5- and 30-month-old male and 30-month-old female B6C3F1 mice. Comparisons of the differences between young and old mice and between old male and female mice were also made.
Materials and Methods

Animal Husbandry and Feeding Regimens

B6C3F1 mice obtained from the National Center for Toxicological Research breeding colony were used. The housing of animals and feeding regimens have been described previously (24, 26). Two groups of mice were used. A group of control mice was fed a standard NIH-31 diet ad libitum (AL). A DR group received 60% of the AL consumption and was fed at 1100 hr daily starting at 16 weeks of age until sacrificed at 5 months (1 month after initiation of DR for young mice) or at 30 months (26 months after initiation of DR for old mice) of age. All mice were given water ad libitum. The control mice for both young and old groups were sacrificed at 5 or 30 months of age. The DR mice received supplemental vitamins to approximate the same level of vitamin intake as in control mice.

Tissue Preparation

Mice were sacrificed between 1300 hr and 1400 hr (12 hr after room lights were turned on) by asphyxiation using carbon dioxide. A small piece of liver tissue from the median lobe of each mouse was sliced into 1-mm thick sections to allow for complete immersion of tissue in a buffer solution of citrate-sucrose-dimethylsulfoxide (33). The tissue was then immediately frozen in liquid nitrogen and stored in a −80°C freezer until assayed.

Hepatic Nuclear Ploidy Classes Measured by Flow Cytometry

Styles (34) described the measurement of ploidy and cell proliferation in the rodent liver by using bromodeoxyuridine. To measure hepatic nuclear ploidy classes in mice, a modified method of Vindelov et al. (33) was used for staining nuclei. A nuclear suspension of the liver tissue was prepared from frozen tissue as described previously (26). Briefly, liver was minced with fine scissors in buffer solution, and nuclei were isolated by trypsinization of the tissue suspension. The nuclear DNA was stained with propidium iodide (PI). The prepared samples were kept at 4°C and analyzed by flow cytometry within 2 hr of staining.

Cellular DNA content was measured using a FACScan Flow Cytometer (Becton Dickinson, San Jose, CA) equipped with an Argon laser illuminating at 488 nm to excite the DNA-associated PI. PI fluorescence was detected at 630 nm. Histograms of 10,000 nuclei were recorded for each sample scanned at a flow rate of 100-200 nuclei/sec. Data were collected using the Consort 30 Data Acquisition Program (Becton Dickinson) and an HP310 Computer (Hewlett Packard, Palo Alto, CA). The DNA histograms were analyzed for the percentage of nuclei containing various amounts of DNA content that represented the percentage of nuclei with different classes of hepatic nuclear ploidy.

DNA histograms, which were analyzed for the percentage of nuclei with various amounts of DNA content, represent the percentage of nuclei with different classes of hepatic nuclear ploidy. To assure having as many nuclear ploidy classes as possible, the voltage of the flow cytometer was set to bring the first peak of diploid (2N) nuclei to channel 30. Under this condition, higher ploidy classes can be identified in higher channels. Three peaks were observed representing the 2N, 4N, and 8N classes of nuclear ploidy. The number of nuclei in each class was represented by the areas under each peak. The number of nuclei in each class was assessed by setting electronic gates about each peak at channels 42-57, 84-103, and 159-182 for diploid, tetraploid, and octaploid populations. There was no other peak appearing beyond channel 182, indicating that no other class of ploidy higher than octaploid was observed. The total percentage of ploidy in the young and old mice of both AL and DR groups were about 75 and 85%, respectively (Table 1). The remaining 15-25% of the total particle count is cellular debris and aggregates of nuclei with debris.

Statistical Analysis

All numerical data were expressed as means ± SEM. Data were compared using the unpaired Student’s t-test (two-tailed). A p-value of <0.05 was considered a statistically significant difference.

Results

Hepatic Nuclear Ploidy Classes between AL and DR Male Mice

Table 1 summarizes the effect of DR on the distributions of hepatic nuclear ploidy of young and old male mice, both in AL and DR groups. The percentages of diploid, tetraploid, and octaploid nuclei in the AL group were comparable with those in the DR group in both young and old male mice.

Effect of Age on Hepatic Nuclear Ploidy Classes in B6C3F1 Mice

The effect of age on hepatic nuclear ploidy classes in young and old male mice is shown in Table 1. The percentage of tetraploid nuclei in old males both in the AL and DR groups was significantly higher (p < 0.05) than that in young males. The same trend was found in octaploid nuclei of old male mice. Thus, a significant increase was detected in the tetraploid and octaploid classes in old males in both AL and DR groups.

Effect of Sexes on Hepatic Nuclear Ploidy Class in B6C3F1 Mice

The effect of sex on hepatic nuclear ploidy classes was significant (Table 2). In the AL group, there was a
significant decrease in diploid and a significant increase in octaploid nuclei in old females versus old males. In the DR group, there was a significant decrease in diploid and a significant increase in tetraploid nuclei in old females versus old males (Table 2).

There were no significant differences in diploid nuclei between AL and DR old female mice. The tetraploid class was found to be increased in the old DR female mice when compared to old AL female mice (29 versus 36%). A decreased octaploid class was found in the old DR females when compared to AL females (18 versus 11%). However, if tetraploid and octaploid classes were pooled as a polyploid class, both old AL and DR female mice had the same proportion (47%) of polyploid nuclei.

Discussion

The S-phase cell population is commonly used as an index of proliferation (31). In liver tissue with a large number of polyploid cells (32), S-phase percentages are difficult to determine. The S-phase cells exist between the diploid (2N) and tetraploid (4N) populations. It is not known whether S-phase cells occur between tetraploid and octaploid (8N) or between octaploid and hexadecaploid (16N).

Measurement of nuclear ploidy classes has been used to measure proliferative activity in the liver (34). S-phase cells in mouse liver stochiometrically stained with PI can be measured by flow cytometry, without interference from changes in ploidy, only from animals less than 1 month old (35).

The present data obtained by flow cytometric analysis show that in the AL B6C3F1, male mice older than 5 months, three different classes of nuclear ploidy were found: diploid, tetraploid, and octaploid. Similar findings of mouse hepatic ploidy have been reported by previous investigators (6,35-36). However, in 6-month-old NMRI mice, a 4% hexadecaploid class was found (35,36). In both DBA and NZB strains of mice, the percentage of hepatic nuclear ploidy class of hexadecaploid was small (36). Thus, the appearance of hexadecaploid nuclei in mouse liver may be strain related.

It is of interest to note that in liver, there are variations in polyploidy between whole hepatic cells and nuclei (6). This is mainly due to the presence of binucleated cells (12). Preparations of intact hepatic cells would show a higher number of cells in higher ploidy classes than preparations of isolated nuclei. The DNA histogram of whole hepatic cells does not reflect true cellular ploidy distributions, simply because the flow cytometer is not able to distinguish between single cells and small aggregates (12).

Our data indicate that the young and old DR mice also exhibit three classes of hepatic nuclear ploidy, similar to AL mice. The percentage of nuclei in diploid, tetraploid, and octaploid was consistent in both AL and DR groups. The pooled percentage of total polyploid nuclei (tetraploid + octaploid) was found to be the same between the AL and DR groups in males. These results indicate that DR did not cause any alterations in total liver nuclear ploidy distributions in males. In females, the tetraploid class was increased and the octaploid class was decreased in DR versus AL mice. Whether this effect is a result of hormones is not known. However, estrogens have been reported to affect the distribution of octaploid nuclei during the estrous cycle in mice (37), with major changes seen in the octaploid nuclei (38). DR is known to decrease estrus cycling and associated hormones in mice (18,20).

Table 1. Comparisons of hepatic nuclear ploidy classes as a function of diet and age in male mice.*

| Ploidy classes | Young | | Old | |
|---------------|------|------|------|------|
|               | AL   | DR   | AL   | DR   |
|               | (n = 6) | (n = 6) | (n = 6) | (n = 5) |
| Diploid       | 43.4 ± 2.4 | 41.7 ± 2.2 | 41.3 ± 1.2 | 40.3 ± 2.8 |
| Tetraploid    | 28.2 ± 1.4** | 27.9 ± 2.1† | 32.9 ± 1.1* | 32.2 ± 1.4† |
| Octaploid     | 3.6 ± 0.4** | 3.8 ± 0.2† | 12.1 ± 0.9** | 12.6 ± 2.1† |

Abbreviations: AL, ad libitum group; DR, dietary restriction group.

*All values are expressed in percent (mean ± SEM); values with at least one similar superscript are significantly different from each other (p < 0.05).

Table 2. Comparisons of hepatic nuclear ploidy classes in old mice as a function of sex with different diet regimens.*

| Ploidy classes | Male | Female | Male | Female |
|---------------|------|--------|------|--------|
|               | (n = 6) | (n = 5) | (n = 5) | (n = 5) |
| Diploid       | 41.3 ± 1.2* | 21.1 ± 2.0* | 40.3 ± 2.8† | 34.2 ± 1.7† |
| Tetraploid    | 32.9 ± 1.1 | 29.4 ± 3.6§ | 32.2 ± 1.4* | 36.7 ± 1.5§* |
| Octaploid     | 12.1 ± 0.9** | 17.9 ± 2.6** | 12.6 ± 2.1 | 10.9 ± 1.1† |

Abbreviations: AL, ad libitum group; DR, dietary restriction group.

*All values are expressed in percent (mean ± SEM); values with at least one similar superscript are significantly different from each other (p < 0.05).
An age effect on the distribution of hepatic nuclear ploidy was observed in both AL and DR groups. When compared with 5-month-old mice, tetraploid nuclei increased 14% and 16% for AL and DR 30-month-old male mice, respectively. Similarly, when compared to young male mice, a 70% increase in octaploid class was observed in both AL and DR old male mice. The pooled percentages of polyploid nuclei in young male mice were 31.8 and 31.0 in AL and DR groups, respectively (Table 1). Similarly, the pooled percentages of polyploid nuclei in old male mice were 45.0 and 44.8 in AL and DR groups, respectively (Table 1). Hepatic ploidy development in mice takes place primarily from birth to 60 days of age in females and to 100 days of age in males. It is reasonable to assume that if DR has an effect, it will be at this time (unpublished data).

It has been reported that the radioresistance of silk-worm embryos increased with the degree of polyploidy (39) during the developmental stages. Increase in ploidy in animals reduces sensitivity to ionizing radiation (40). This fact suggests that duplication of genome copies may be an adaptation to protect against DNA damage (40). In polyploid liver cells, if one genome copy becomes damaged by radiation or exposure to carcinogens, the cell would be protected and not suffer irreversibly due to the capability of additional genomic copies to provide active gene products. Polyploidization in mammalian liver cells may reduce the rate of onset of deleterious changes so that the cells may achieve a long life span and thus contribute to the survival of the animal. The increase in polyploidy with age seen here is consistent with this idea, if one considers increasing polyploidy as an adaptive or protective change that occurs in aging.

It can be concluded from our results that hepatic nuclear polyploidization in male mice is already established by 5 months of age. This is also true for DR groups even though the liver is much smaller. DR started after 5 months of age does not alter ploidy distributions in males. Interestingly, DR started at this age significantly decreases the incidence of spontaneous liver tumors in males (21,24) at 30 months of age. However, DR started at 4 months of age for 26 months had no effect on ploidy changes. This suggests that the mechanism by which adult-onset DR inhibits liver tumors is not related to a change in polyploidy. In DR females, a shift of percentages of nuclei in tetraploidy (increased) and octaploidy (decreased) was observed. It is not known if DR started before 4 months of age would have an effect on hepatic nuclear ploidy distribution. Further study using earlier DR, starting right after weaning and before 4 months of age, is warranted.

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