RADIOAUTOGRAPHIC STUDY ON THE INCORPORATION OF TRITIATED THYMIDINE BY MOUSE LIVER CELLS DURING RESTITUTION AFTER STARVATION

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Received for Publication October 17, 1969

DNA synthesis has been studied on the liver cells of mice in the restitution period after starvation. Tritiated thymidine (³H-TdR) was subcutaneously injected twice a day for ten days during the restitution period, in order to examine the incorporation of ³H-TdR into hepatic nuclei and the persistence of ³H-labelled nuclei in the liver.

After ten-day's labelling, about 40% of hepatic parenchymal cell nuclei was heavily labelled. More incidence of heavily labelled nuclei was observed at 1 week after the last injection of ³H-TdR. Thereafter, the percentage of heavily labelled nuclei remained nearly constant around 30%. If lightly labelled nuclei were taken into account, almost 80% of the parenchymal cell nuclei showed labelling of DNA. With exception at 1 week after the last dose of ³H-TdR, this total percentage of labelled nuclei remained unchanged about 60% for 6 weeks.

The incorporation of ³H-TdR into littoral cells of the liver was also observed. Throughout the present experimental period from 35 to 40% of littoral cells had labelled nuclei.

These results indicate that DNA synthesis has occurred in the parenchymal and in the littoral cell nuclei during the restitution after starvation, and that the nuclei, which contain newly synthesized DNA, persist for a long period.

Although in the liver of the full grown mammal mitotic activity has normally ceased, the tissue is capable of rapid growth by mitosis.

Many factors are reported to affect liver regeneration (11); among these regeneration after removal of a part of the organ has been most extensively studied (3, 5, 14). After the partial hepatectomy in the rat, mitotic activity rises abruptly reaching a peak at about 38 hours, at which time the number of mitoses per 100 parenchymal cells is estimated to be from 4 to 5 (5, 14). As regard DNA synthesis in these cells it is reported that the incidence of tritiated thymidine (³H-TdR) incorporation by them is elevated more rapidly after partial hepatectomy and 24 hours thereafter nearly 40% of the parenchymal nuclei become to be labelled radioautographically (5).

In the present experiment, a high incidence of ³H-TdR incorporation by hepatic

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parenchymal cells, comparable to that observed after partial hepatectomy, was observed in the adult mouse during the recovery period after starvation. Although an increase in the mitotic activity of the mouse liver during restitution after starvation has been reported by Leduc (9), little is known concerning DNA synthesis of the hepatic parenchymal cells under this condition.

MATERIAL AND METHODS

Adult male mice of DBA-strain, about three months old, were deprived of food for 5 days, and then they were reared on the Oriental compressed diet for mice (Oriental-MF, Japan) containing 24.2% of protein. They were allowed to free access to water throughout the experiment.

They were subcutaneously injected with $5\mu\text{Ci}$ of ^3H -TdR (specific activity : 2.7 Ci/mM)* twice a day for the first 10 days of recovery period except the last day when $10\mu\text{Ci}$ of the precursor was administered. At 4 hour, 1 week, 2 weeks, 4 weeks, and at 6 weeks after the last injection of the precursor, two mice were sacrificed respectively. Livers were removed, fixed for several hours in Carnoy's fluid, and embedded in paraffin. The sections of 5 to 7μ thick, were covered with Kodak AR-10 stripping film. After being exposed under standard, dry conditions at 4°C for 62 days, the radioautographs were developed in Kodak D 19b for 5 minutes at 20°C and ultimately stained through the gelatin film with Mayer's hematoxylin.

In order to certify the incorporation of the precursor into DNA, DNase digestion of the sections was carried out for 24 hours at 37°C in the enzyme solution containing 0.05 mg crystallized Worthington deoxyribonuclease per ml of Gomori's Tris buffer with 0.2 M MgSO_4 (1), before covering the sections with stripping film. To remove RNA from liver cells, sections were incubated in the 10% solution of perchloric acid for 16 hours at 4°C .

RESULTS

Multiple injections of ^3H -TdR during the recovery period after starvation resulted in an accelerated incorporation of the precursor by liver cells. Figures 1 and 2 show the heavily labelled hepatic parenchymal nuclei. The maximum numbers of grains per nucleus were in the range of from 200 to 300. These labelled cells were more than 60% for the liver parenchymal cells and about a half of the labelled cells had more than 30 grains per nucleus. These cells were designated as heavily labelled. It should be noted here that littoral cells, or stellate cells of Kupfer and endothelial cells of sinusoid, were also labelled; the maximum numbers of grains being around 20 per nucleus. In either parenchymal cells or littoral cells silver grains were located exclusively on the nucleus. After DNase digestion of the sections prior to radioautography, all the silver grains on the cells disappeared completely. On the other hand, the pretreatment of the sections with cold PCA, which removed almost all RNA, caused no significant changes in the numbers of the silver grains on the cells.

Figures 3 and 4 show the labelled hepatic cells 4 hours after the last dose of

* Obtained from the Radiochemical Centre, Amersham, England.

Fig. 1. Autoradiographs of a section of the liver of a mouse killed 4 hr after the last injection of ^3H -TdR, showing heavily labelled parenchymal cells. Five microcurie of ^3H -TdR was administered twice a day for the first 10 days during the recovery period after starvation. Exposure time 62 days. Magnification 1,000.

Fig. 2. Autoradiographs of a liver section 4 hr after ^3H -TdR. Heavily labelled parenchymal cells, numbers of grains being around 300, and lightly labelled littoral cells (indicated by arrows) are seen. Exposure time 62 days. Magnification 1,000.

Fig. 3. Autoradiographs of a section of a mouse liver killed 4 hr after ^3H -TdR, showing a high incidence of labelled parenchymal cells. Exposure time 62 days. Magnification 400.

Fig. 4. Autoradiographs of a section of the liver of a mouse killed 4 weeks after the last injection of ^3H -TdR. As many labelled parenchymal cells are seen as are in the figure 3. Exposure time 42 days. Magnification 400.

Fig. 5. Histogram showing percentages of labelled hepatic parenchymal cell at various times after the $^3\text{H-TdR}$ administered during the recovery period after starvation. Counts were performed at least on 400 cells at a given time.

Fig. 6. Histogram showing levels of labelled littoral cells of the liver at various times after $^3\text{H-TdR}$.

$^3\text{H-TdR}$ and those after 4 weeks respectively. It is seen that as many cells are labelled at 4 weeks as 4 hours after the injection of $^3\text{H-TdR}$. The number of the labelled cells were counted at appropriate intervals, and the results were summarized in figures 5 and 6.

Figure 5 represents the results obtained in the parenchymal cells. About 76% of the liver parenchymal cells were labelled and about a half of these cells had more than 30 grains per nucleus. It is worthy of note that the proportions of the labelled cells were nearly constant, about 60%, throughout the present experiment except at 1 week. The ratios of the heavily labelled cells to the whole labelled cells were also in the same order of about 1 : 2.

As shown in figure 6, about 40% of the liver littoral cells incorporated $^3\text{H-TdR}$. The labelled cells persisted for 4 weeks without significant changes either in the labelling index or in the number of silver grains per nucleus. Six weeks after the labelling, however, the incidence of labelled cells decreased to 20%.

Control mice which were injected with the same amount of $^3\text{H-TdR}$ alone without fasting, had the liver in which only about 5% or less cells were labelled with the DNA precursor.

So far as the present experiment is concerned, neither increase in the number of mitotic figures nor necrosis, which might be responsible for the accelerated DNA synthesis, was found at any time and in any part of the liver during restitution after starvation.

DISCUSSION

From the results of the present experiment, it is evident that the incorporation of ^3H -TdR by hepatic parenchymal cells in the adult mouse is greatly increased by refeeding after starvation. It is interesting to note that the labelling indices of parenchymal cells were above 60% and much higher than those (around 40%) observed after partial hepatectomy (3, 14). This might be ascribed to multiple injections of ^3H -TdR during restitution period after fasting. However, further study to be reported elsewhere revealed that a single dose of ^3H -TdR often produces a high incidence of labelled parenchymal cells similar to that observed after multiple injections.

The incidence of labelling of littoral cells lining the sinusoids with ^3H -TdR also increased during restitution after fasting, but to a lesser extent as compared with that of parenchymal cells. In addition, it should be emphasized here that, while the maximum numbers of grains in the parenchymal cells varied from 200 to 300 per nucleus, the corresponding numbers in the littoral cells were as low as 20 per nucleus. This is attributable to the degree of polyploidy of parenchymal cells. According to Epstein (6), polyploidy of these cells increases with age and hepatic parenchymal cells with 8N and 16N begin to increase in number. The degree of polyploidy in the parenchymal cells appeared to increase to some extent by refeeding after starvation, because the number of grains per nucleus in the parenchymal cells was unexpectedly much greater than that in the littoral cells which are known to have no polyploidy. Since no cell counts with respect to polyploidy were performed, however, in the present study, a further statistical analysis is to be taken to settle the problem.

Changes in the mitotic activity of hepatic parenchymal cells in relation to refeeding after starvation must next be considered. It is reported that at a given instant about 10 times as many nuclei are labelled as are undergoing mitosis (13), since DNA synthesis requires approximately from 6 to 8 hours for completion whereas mitosis only from 30 to 45 minutes. The data mentioned in the introduction also show that the incidence of ^3H -TdR incorporation by hepatic parenchymal cells is about 10 times as high as that of mitosis during regeneration after partial hepatectomy. If we assume that the conditions in the liver during restitution after starvation were quite similar to those during regeneration of the organ after partial hepatectomy, the mitotic index of hepatic parenchymal cells should be around 6%, because the labelling index of these cells was 60%. As far as the present experiment is concerned, however, such a high mitotic activity of parenchymal cells was not observed at any time and in any part of the liver. Nonetheless, a more detailed study is wanted to clarify the question whether the hepatic parenchymal cells are really non-mitotic during the restitution after starvation as reported by many investigators (2, 4, 7, 8, 12) or not, since Leduc (9) has reported that mitotic activity of both parenchymal and littoral cells in the mouse liver is enhanced by starvation and refeeding.

Another point of interest is the finding that the labelled cells, both of parenchymal and littoral, persisted for a considerable long period and that the number of grains per nucleus remained almost unchanged. This suggests a long lifespan of both types of cells without mitotic division. It is reported that hepatic parenchymal cells have a lifespan of approximately 3,000 days (10).

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