Effect of Age on the Formation of Small-Cell Colonies in Cultures of Primary Rat Hepatocytes

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ABSTRACT

The proliferation of primary cultured rat hepatocytes was observed in serum-free modified Dulbecco's modified Eagle's medium supplemented with 10 mm nicotinamide and 10 ng/ml of epidermal growth factor. These proliferating cells were mainly mononucleate and formed small-cell colonies after Day 4. The small cells in focal colonies were surrounded by typical hepatocytes and were stained immunocytochemically with anti-rat albumin and anti-cytokeratin 8 antibodies. This suggests that the cells in the small-cell colonies were derived from hepatocytes. The frequency of appearance of small-cell colonies with age was examined by the use of primary cultured hepatocytes isolated from the livers of rats between the ages of 3 wk and 90 wk. In the cells from 4- to 5-wk-old rats, about 58 colonies per 1000 attached cells appeared 144 h after plating; the number of colonies rapidly decreased to about 25 in 6- to 8-wk-old rats. In adult rats, about 17 colonies were seen, and only about five colonies were observed in rats more than 80 wk old.

INTRODUCTION

We previously reported that the proliferation of primary cultured rat hepatocytes was observed in serum-free medium supplemented with 10 mm nicotinamide and EGF (1) and that the proliferating cells were mainly mononucleate and formed small-cell colonies after Day 4 (2). Colony formation of primary cultured hepatocytes was also observed in L-15 medium supplemented with EGF (3), and it also occurred in mouse hepatocytes (4). Furthermore, cells in small-cell colonies were shown immunocytochemically to possess albumin and transferrin in their cytoplasm and ultrastructurally to have rich mitochondria and a few peroxisomes (2, 5). These facts indicate that the cells in the focal colonies were derived from hepatic parenchymal cells. In addition, we suggest that, in adult rat livers, if a colony is derived from one cell, about 1.5% of the hepatocytes might have the potential of multiple replication and of forming a separate colony (2). In the present experiment we examined the frequency of appearance of small-cell colonies in primary cultured hepatocytes during the aging of rats.

MATERIALS AND METHODS

Isolation and Culture of Rat Hepatocytes. Twenty-six male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were used to isolate hepatocytes by the two-step liver perfusion method of Seglen (6), with some modifications (1). The details of the isolation and culture procedures were previously described (2). The viability of the isolated cells was examined by the trypan blue exclusion test (more than 95% viable in this experiment). The cells were suspended in modified L-15 medium. Hepatocytes were plated on dishes (Corning Glass Works, Corning, NY) coated with rat-tail collagen (0.5 g of dried tendon/filter of 0.1% acetic acid) (7) and were placed in a 100% air incubator at 37°C. Two to 3 h after plating, the medium was changed to modified DMEM (Kyoritsu Pharmaceutical Co., Tokyo, Japan) (2) supplemented with 10 ng/ml of EGF (Collaborative Research, Inc., Lexington, MA); 25 mm NaHCO3, and 10 mm nicotinamide (Katyama Chemical Co., Osaka, Japan). The cells were then placed in a humidified, 5% CO2/95% air incubator at 37°C. The medium was replaced with fresh medium every other day.

Immunocytochemical Examinations. The cells were fixed with cold absolute ethanol. For immunocytochemistry, rabbit anti-rat albumin (Cappel, Malvern, PA) and mouse anti-cytokeratin 8 and 18 (Amersham International, plc., England) antibodies were used as primary antibodies. We used the ABC method for immunocytochemistry. To carry out the double stainings (albumin + BrdUrd or cytokeratin 8 + BrdUrd), Vector Red, and 3,3'-diaminobenzidine were used as the substrates for alkaline phosphatase staining (Vectastain alkaline phosphatase kit; Vector Laboratories, CA) and for peroxidase staining (Vectastain ABC elite kit; Vector Laboratories, respectively). Anti-rat albumin antibody did not react with bovine serum albumin of the culture medium as shown by Western blot analysis. Nonparenchymal cells in the cultures were used as a negative control.

Colony Counts. We used four 35-mm dishes of each cell density (6 × 105 cells or 9 × 105 cells/dish) per experiment. Two of the dishes were treated with 40 μM BrdUrd (Sigma Chemical Co., St. Louis, MO) from 24 to 48 h after plating, and the others were similarly treated from 96 to 144 h. The former and the latter dishes were fixed in cold absolute ethanol at 48 h and 144 h, respectively, after three rinses with cold phosphate-buffered saline. Immunocytochemistry for BrdUrd was carried out with mouse anti-BrdUrd antibody (DAKOPATTS a/s, Copenhagen, Denmark) as primary antibody, followed by the ABC method. The definition and the method of counting small-cell colonies were previously described (2).

RESULTS

In order to examine whether the frequency of appearance of small-cell colonies decreases with increasing age, we used primary cultured hepatocytes isolated from 3- to 90-wk-old rats. We previously reported that the incidence of colonies depended on the plating density of hepatocytes (2). The best incidence was observed in young adult rats when 600,000 cells were plated on a 35-mm dish. However, since the isolated cells from 3- to 6-wk-old rats were smaller than the cells from older rats, we examined two densities (6 × 105 cells or 9 × 105 cells/dish) of cells in each experiment. In the 3- to 6-wk-old rats, the best incidence of colonies was observed when 9 × 105 cells were plated on the dishes. On the other hand, in rats more than 8 wk old, the best incidence resulted from a plating density of 6 × 105 cells. The same as previously reported (2). The cells isolated from a 3-wk-old rat were very small, and more than 90% of the cells were mononucleate. To prove that the proliferating cells were hepatic parenchymal cells, we carried out the immunocytochemistry for albumin and for cytokeratin 8 and 18, which were expressed in hepatocytes in vivo. As shown in Fig. 1A, the cytoplasm of most cells was stained with anti-rat albumin antibody. Furthermore, those cells also expressed cytokeratin 8 (Fig. 1B) and 18 (data not shown). However, as shown in Fig. 2A, because more than 70% of the cells were labeled with BrdUrd between 96 and 144 h after plating, we could not identify the cells as a colony. In the hepatocytes from 4-wk-old rat (Fig. 2B), labeled cells looked like beginning colonies, and it was possible to count the

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3 The abbreviations used are: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; BrdUrd, 5-bromo-2′-deoxyuridine; ABC, avidin:biotinylated enzyme complex.

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SMALL-CELL COLONIES OF RAT HEPATOCYTES DURING AGING

Fig. 1. Immunocytochemical studies in primary cultured hepatocytes isolated from a 3-wk-old (A, B) and a 14-wk-old rat (C, D). A, immunocytochemistry for albumin. Cells \((9 \times 10^5)\) were plated on the dish and fixed with cold absolute ethanol at 144 h. An arrow shows dead cells. \(\times 330\). B, immunocytochemistry for cytokeratin 8. Cells \((9 \times 10^5)\) were plated on the dish and fixed at 144 h. Arrows show nonparenchymal cells, which are not stained with cytokeratin 8. \(\times 330\). C, double immunocytochemistry for albumin and BrdUrd of the small cells in focal colonies. Forty \(\mu M\) BrdUrd was added to the medium between 96 and 144 h after plating. Cells \((6 \times 10^5)\) were plated on the dish and fixed at 144 h. Darkened nuclei are those stained with BrdUrd. Cells with darkened cytoplasm are those stained with albumin. Small mononuclear cells in the focal colonies surrounded by arrowheads are stained with both albumin and BrdUrd. \(\times 235\). D, double immunocytochemistry for cytokeratin 8 and BrdUrd of the small cells in focal colonies. Forty \(\mu M\) BrdUrd was added to the medium between 96 and 144 h after plating. \((6 \times 10^5)\) were plated on the dish and fixed at 144 h. Darkened nuclei are those stained with BrdUrd. Cells with darkened cytoplasm are those stained with cytokeratin 8. Small mononuclear cells in the focal colonies surrounded by arrowheads are stained with both cytokeratin 8 and BrdUrd. \(\times 330\).

number of colonies. Compared with the cells from adult rat hepatocytes (Fig. 2C), the sizes and features of the cells in the colonies were similar to normal hepatocytes, although the sizes of the surrounding cells were quite different. The cells in small colonies, which were isolated from either 4- to 6-wk or more than 8-wk-old rats, were stained with anti-albumin antibody (Fig. 1C) and cytokeratin 8 (Fig. 1D) antibody. On the other hand, the labeling indices of cells isolated from aged rats were much lower than those of cells from the older rats (data not shown). The low labeling index and \([\text{3H}]\)thymidine uptake into DNA by aged rat hepatocytes were also reported in primary cultures of female SD rat hepatocytes (8). Inasmuch as some hepatocytes under these culture conditions were dead and detached from the dish after day 4, empty areas were often observed in the dishes at the time of fixation. Many relatively large mononucleate and binucleate hepatocytes did not take up BrdUrd into their DNA (Fig. 2D). Mainly small mononucleate cells were labeled. However, although the incidence of colonies was low, the sizes and features of cells in focal colonies did not show any differences from those of cells from younger rats.

As shown in Fig. 3, the frequency of appearance of colonies at 144 h as a function of those cells attached at 48 h was investigated with increasing age of rats. In 4- to 5-wk-old rats 58.8 ± 2.8 colonies per 1000 attached cells appeared; the number of colonies rapidly decreased to 25.8 ± 3.6 colonies in 6- to 8-wk-old rats. The number was stable in 10- to 15-wk-old rats (17.4 ± 3.3 colonies), and, thereafter, the number gradually decreased with age. Only 5.5 ± 1.1 colonies were observed in rats more than 80 wk old.

DISCUSSION

We previously demonstrated that primary cultured hepatocytes isolated from normal adult rats could proliferate in serum-free medium supplemented with nicotinamide and EGF (1, 2). Under these culture conditions, cells smaller than normal hepatocytes with a single nucleus appeared after the hepatocytes had divided once or twice, and these small cells formed small focal colonies after Day 4 or 5 in culture (2). Furthermore, these small proliferating cells appeared to be diploid by flow cytometric analysis (2). In addition, the cells in focal
colonies were immunocytochemically stained with hepatic secreting proteins such as albumin and transferrin. Ultrastructurally these cells were shown to possess many mitochondria and a few peroxisomes (2, 5). In the present study the cells in focal colonies that were isolated from rats of various ages also had albumin and cytokeratins in their cytoplasm. These proteins were also expressed even in the small proliferating cells from a 3-wk-old rat. These results indicate that the small proliferating cells forming colonies are derived from hepatic parenchymal cells.

In our previous paper we suggested that two kinds of hepatocytes existed in an adult rat liver (2). One consists of hepatocytes whose main function is to maintain the differentiated functions, whereas the other may be a type of stem cell having the potential for both differentiated functions and replication. However, in view of the present results, hepatocytes may be classified into three types of cells with respect to their ability to divide. (a) Type I cells that have a high potential to proliferate, form colonies, and continue to supply daughter cells. These cells are usually mononucleate and diploid (2). (b) Type II cells are cells in which the number of possible cell divisions is limited to a few divisions; these cells normally express the fully differentiated functions. (c) Type III cells lose the ability to divide and reach the final differentiated state. On the basis of this classification of hepatocytes, we can interpret liver growth and the proliferation of parenchymal cells as follows. In young rats, many hepatocytes are type I cells and, with growth, many of these cells become type II cells, thus decreasing the number of type I cells. In this period both type I and type II cells divide, and this activity contributes to the rapid increase in the total number of parenchymal cells. With increasing age of rats, some type II cells complete their role of proliferation and become type III cells, and, after completing their role as differentiated hepatocytes, type III cells will die (apoptosis). In normal adult rat liver, to compensate for the eliminated cells by death or other reasons, type I cells may produce new cells. The existence of progenitor cells, which continuously produced new cells, and cell streaming from the portal area to the pericentral area were suggested by some investigators (9-11). They reported that the progenitor cells were in the periportal area and that the cells' graveyard was located at the tissue periphery adjacent to the terminal hepatic vein. Furthermore, they classified the liver acinus into two compartments: proliferating and nonproliferating. The former was in the inner third of the acinus, and the latter was in the other two-thirds. However, the in vivo experiment of continuous administration of [3H]-thymidine to rats showed that...
99% of hepatocytes in a weaning rat, 93% of cells in a young adult rat, and only 77% of cells in a 2.5-yr-old rat synthesized their DNA during regeneration after two-thirds partial hepatectomy (12). This result means that most hepatocytes can proliferate whenever the lost hepatocytes must be rapidly replaced. Thus, with respect to the ability of dividing, the existence of three rather than two types of hepatocytes seems plausible. Type II cells may occupy the major parts of the acinus. With aging, the ratio of type II cells to type III cells will change; thus, the number of type III cells increases with age. This is supported by the facts that some cells never take up BrdUrd into their DNA in culture and that the number of cells labeled by continuous incorporation of [3H]-thymidine into DNA in aged rat hepatocytes (14) are much lower than in the young rat, and DNA synthesis proceeds more slowly even though the livers finally restore their original mass (14, 15). These results may indicate that the population of the cells that can proliferate decreases in aged rat livers and that the limited number of proliferating hepatocytes goes through more cycles to accomplish regeneration (16).

The existence of hepatic stem cells or progenitor cells is still being argued, and the definition of these cells is now confusing. At present, as described by Fausto (17), if the stem cells are defined as undifferentiated, multipotent cells and the progenitor cells as being nonhepatocyte epithelial cells, capable of dual lineage generation (biliary and hepatocytic), and if both of these lineages express hepatocyte markers, no pure population of either type of cells has been isolated from normal, injured, or preneoplastic adult livers. One possible candidate for progenitor cells, oval cells, which appear during hepatocarcinogenesis and chemically induced severe liver injury, has been proposed by many investigators. Recently, oval cells have been shown to possess bipotential of differentiating either toward biliary or hepatocellular lineage (18-24, reviews). However, the identification of the progenitor cells in normal adult livers has not been achieved, and they do not expand initially in regeneration or after most injuries (25). Furthermore, no in vitro systems have yet been developed where full morphological and functional differentiation of the progenitor cells into hepatocytes occurs. The small cells (type I cells), which we showed in the previous (2, 5) and present experiments, seem to fit into neither the stem cell nor progenitor cell classification, because these small cells appear morphologically and functionally to be true hepatocytes, although the cells are less differentiated. Therefore, these cells may be considered as "committed progenitor cells" that can further differentiate into hepatocytes. Although it is difficult to identify the location of the cells that can form the small-cell colonies in normal rat livers, we suggest that cells possessing a high potential to proliferate exist in normal rat livers and that the small-cell colony formation is dependent on the age of the rat.

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Fig. 3. Scatterplots of the relationship between the number of the small-cell colonies and the age of rats. Twenty-four rats were examined. In the 3- to 6-wk-old rats, 9 × 10⁷ cells were plated on the dishes and, in rats more than 8 wk old, 6 × 10⁷ cells were plated. A total of 40 μM BrdUrd was added to the culture medium from 96 to 144 h. At 144 h cells were fixed, and immunocytochemistry for BrdUrd was conducted. The small-cell colonies that consisted of more than eight mononucleate cells stained with BrdUrd were counted. The frequency of appearance of the colonies (ordinate) was calculated as the number of the colonies at 144 h per 1000 attached cells at 48 h after plating.

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