TGF- β Completely Blocks the Formation of Small-Cell Colonies: Effects of Mito-inhibitory Factors on the Proliferation of Primary Cultured Rat Hepatocytes

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ABSTRACT. The proliferation of primary cultured rat hepatocytes was observed in serum-free modified DMEM supplemented with 10 mM nicotinamide and 10 ng/ml EGF. These proliferating cells were mainly mononucleate and formed small-cell colonies after 4 days of culture. In the present experiment primary cultured hepatocytes were treated with Activin A, IL-1 β , IL-6, and TGF- β , which have been shown to be inhibitors of the DNA synthesis of rat hepatocytes, to examine whether these four inhibitors could suppress the formation of small-cell colonies. The initial DNA synthesis of more than 50% of the cells was dose-dependently inhibited by all the factors and the strongest inhibition was demonstrated in the cells treated with TGF- β . Although Activin A and IL-6 did not block the colony development when the agents were administered at 96 h, just before the time when the cells started to form colonies, TGF- β and IL-1 β could inhibit the colony formation completely and partially, respectively. Transient treatment (48–72 h) with TGF- β was enough to suppress colony development, while Activin A and IL-6 did not block the formation of colonies. IL-1 β partially suppressed this formation. However, continuous administration (48–144 h) of IL- β as well as TGF- β stimulated the detachment of the cells from dishes and the remaining hepatocytes failed to form colonies. In addition, only TGF- β could inhibit the DNA synthesis of most small cells in the established colonies as well as that of relatively large hepatocytes. Neither Activin A, IL-1 β nor IL-6 could inhibit the DNA synthesis of the small cells. Thus, only TGF- β could completely inhibit both the DNA synthesis of any type of hepatocyte and the formation of small-cell colonies.

We previously reported that the proliferation of primary cultured rat hepatocytes was observed in serum-free medium supplemented with 10 mM nicotinamide and EGF (8) and that the proliferating cells were mononucleate, possessed small cytoplasm, and formed small-cell colonies from day 4 or 5 after plating (9). Immunocytochemically and ultrastructurally these cells had hepatic characteristics (9, 10). These colonies were shown to be induced not only by EGF but also by fibroblast growth factors, hepatocyte growth factor, and TGF- α (11). In addition, the number of the colonies in the cultures was dependent on the age of the rats and the hepatocytes isolated from younger rats formed more colonies in the cultures (12). Taking these results into consideration, we proposed that hepatocytes might be classified into three types of cells with respect to their

ability to divide (11, 12): (a) cells that have a high potential to proliferate and form colonies (Type I cells; committed progenitor cells); (b) cells for which the number of possible cell divisions is limited to several divisions; these cells normally express fully differentiated functions (Type II cells); and (c) cells that lose the ability to divide and reach the final differentiated state (Type III cells).

Growth inhibitors of hepatocytes in primary culture have been investigated in many laboratories. TGF- β was first shown to be a growth inhibitor of rat hepatocytes (1, 7, 13). Nakamura *et al.* (14) showed that cytokines IL-1 β and IL-6 could suppress the DNA synthesis of rat hepatocytes *in vitro*. However, concerning IL-6, the effect on the DNA synthesis of hepatocytes is still controversial because it was shown that IL-6 might stimulate ³H-thymidine incorporation into the DNA of mouse hepatocytes (4). Recently, Activin A, which belongs to the TGF- β gene superfamily, was demonstrated to block EGF-mediated DNA synthesis of rat hepatocytes in primary cultures (24). Furthermore, the cultured hepatocytes were shown to secrete Activin A into the medium, resulting in the suppression of their

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Abbreviations: EGF, epidermal growth factor; TGF, transforming growth factor; IL, interleukin; DMEM, Dulbecco's modified Eagle's medium; LI, labeling index; BrdU, 5-bromo-2'-deoxyuridine; ABC, avidin: biotinylated enzyme complex; HE, hematoxylin and eosin; CK, cytokeratin; Tf, transferrin; Tf-R, transferrin receptor; PBS, phosphate-buffered saline.

DNA synthesis.

In the present study we examined whether each type of hepatocyte, especially Type I and Type II cells, which we defined, could be differently regulated by these four inhibitors with regard to the DNA synthesis and the formation of small-cell colonies.

MATERIALS AND METHODS

Isolation and culture of rat hepatocytes. Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan), weighing 200–400 g were used to isolate hepatocytes by the two-step liver perfusion method of Seglen (19), with some modification (9). Rats were operated on under deep anesthesia by pentobarbital. The details of the isolation and culture conditions were previously described (9). The viability of the isolated cells was examined by the trypan blue exclusion test (more than 90% viable in this experiment). The cells were suspended in modified L-15 medium. Then 6×10^5 cells were plated on 35-mm dishes (Corning Glass Works, Corning, NY) coated with rat-tail collagen and were placed in a 100% air incubator at 37°C. Two to three hours after plating, the medium was changed to modified DMEM supplemented with 25 mM NaHCO₃, 0.5 µg/ml insulin (Sigma Chemical Co., St. Louis, MO), 10^{-7} M dexamethasone (Sigma Chemical Co.), 10 mM nicotinamide (Katayama Chemical Co., Osaka, Japan), and 10 ng/ml EGF (Becton Dickinson Labware, Bedford, MA). The cells were then placed in a 5% CO₂/95% air incubator at 37°C. The medium was replaced with fresh medium every other day except where specifically mentioned.

Mito-inhibitory factors. IL-1β, IL-6, and TGF-β were purchased from Becton-Dickinson Labware, R & D Systems (Minneapolis, MN), and Takara Shuzo Co. (Kyoto, Japan), respectively. Activin A was a generous gift from the Central Research Laboratories of Ajinomoto Co., Inc., Kawasaki, Japan. Each factor was added to the medium at 48–72 h, 96–144 h, or 168–216 h after plating. In the experiments using continuous administration, the factors were added every 24 hrs from 48 h after plating.

Labeling indices and colony counts. For examinations of LI and counts of small-cell colonies, 40 µM BrdU (Sigma Chemical Co.) was added to the culture medium for 24 hrs or 48 hrs before fixation. The cells were fixed in cold absolute ethanol after three rinses with PBS. Immunocytochemistry for BrdU was carried out with mouse anti-BrdU antibody (DAKO, Copenhagen, Denmark) as the primary antibody, followed by the ABC method (ABC Elite Kit, Vector Laboratories, Inc., Burlingame, CA). The cells were counterstained with HE. To determine the LI, 10 fields per dish and 3 dishes per experiment were examined. The definition of a colony and the method of counting the colonies were previously reported (9).

Immunocytochemistry of small-cell colonies. Cells were fixed in cold absolute ethanol. Mouse anti-CK 8 (Amersham International PLC, UK), mouse anti-rat Tf-R (Chemicon,

Temecula, CA), and rabbit anti-rat Tf (Cappel Laboratories, Malvern, PA) antibodies were used as primary antibodies, followed by the ABC method. Non-parenchymal cells in the cultures were used as a negative control.

RESULTS

Primary cultures of rat hepatocytes were treated with 10 mM nicotinamide and 10 ng/ml EGF. As previously reported, the colony formation depended on the concentration of EGF and the maximum number of colonies was observed at 10 ng/ml (11). In addition, we found that the number of colonies decreased with the age of the rats (12). In this experiment we used rats 8 to 14 weeks old. Therefore, the number of the colonies counted varied with the experiment. In these culture conditions the first peak of DNA synthesis of cultured hepatocytes was observed at around 60 h after plating (8). By treating hepatocytes with mito-inhibitory factors before the first DNA synthesis, we confirmed that they could inhibit the DNA synthesis of the hepatocytes. As shown in Fig. 1, Activin A, IL-β, IL-6, and TGF-β could decrease the LI of the hepatocytes. Both Activin and TGF-β were reported to induce apoptosis in primary cultured hepatocytes (15, 16, 18), but, in this experiment, 24-hr treatment with Activin A did not cause cell death at 72 h after plating. However, the number of the cells detached from the dishes increased as the concentration of TGF-β increased to more than 5 ng/ml. Dose responses of the effect of each factor on the DNA synthesis of cells were investigated (Fig. 2). We found that 5 ng/ml IL-β inhibited 40–50% of the DNA synthesis of the cells. More than 10 ng/ml IL-β did not stimulate its effect. IL-6 showed 40–50% inhibition at 10 ng/ml, and even 100 ng/ml did not strengthen the effect. Activin A also exhibited an action similar to that of IL-6. The maximum suppression of the DNA synthesis was observed at 10 ng/ml. The strongest inhibition of DNA synthesis of the cells was shown with the use of TGF-β. More than 60% of the hepatocytes stopped entering the S-phase of the cell cycle. In addition, when the cells were treated with TGF-β for 24 hrs before BrdU addition, only a few labeled nuclei were observed in the cultures (data not shown).

As previously described, the formation of small-cell colonies seems to require 3 to 4 rounds of cell replication of Type I cells (9). Therefore, we next investigated whether the growth inhibitors could suppress the formation of the small-cell colonies. As shown in Fig. 3, small-cell colonies, which consisted of BrdU-positive mononucleate cells and had small cytoplasm, were observed at 144 h after plating. Only TGF-β could completely inhibit the formation of these colonies (Fig. 3E) when the inhibitor was added at 96 h. However, neither Activin A, IL-1β, nor IL-6 showed any effect on the for-

Fig. 1. Immunocytochemistry for BrdU in primary culture of hepatocytes in modified DMEM supplemented with 10 mM nicotinamide and 10 ng/ml EGF (A). 20 ng/ml Activin A (B), 10 ng/ml IL-1 β (C), 10 ng/ml IL-6 (D), or 5 ng/ml TGF- β (E) was added to the cells at 48 h after plating. A total of 40 μ M BrdU was added to the culture medium from 48 to 72 h. Cells were fixed with cold absolute ethanol at 72 h, and the immunocytochemistry for BrdU was carried out and cells were then counterstained with HE. Darkened nuclei are those stained with BrdU. All photos are the same magnification ($\times 118$).

mation (Figs. 3B, C, and D). Even a high concentration of 100 ng/ml of those three agents could not induce the suppression of colony formation. However, they seemed to inhibit the DNA synthesis of the cells surrounding the colonies. Table I shows the present inhibition of the colony formation. Almost complete suppression of the development of the colonies was observed in the cultures treated with TGF- β and the concentration of 2 ng/ml was enough to inhibit it. Neither Activin A nor IL-6 was effective to decrease the number of the colonies. IL-1 β partially suppressed the formation of colonies. The DNA synthesis of the neighboring cells around the colonies might be inhibited by Activin A, IL-1 β and IL-6. Even if a higher concentration of Activin A, IL-1 β , or IL-6 was applied to the cells, similar results were obtained (data not shown).

In the next experiment, to examine whether transient or continuous exposure to each factor could inhibit the formation of small-cell colonies, we treated cells with

each factor from 48 h after plating. The hepatocytes were exposed to each inhibitor between 48 h and 72 h or between 48 h and 144 h after plating. Figure 4 and Table II show the cultures and the number of the colonies observed at 144 h, respectively. The hepatocytes treated with TGF- β transiently or continuously did not develop colonies (Figs. 4D and H). It was of interest that cells with multinuclei and large cytoplasm appeared in the culture (Fig. 4D). Although transient treatment with IL-1 β partially blocked the formation of the colonies, continuous exposure completely inhibited the formation (Figs. 4B and F). Enlargement of the spaces between the cells was observed in the cultures treated with IL-1 β , and continuous addition induced detachment of cells from the dishes. On the other hand, the cells treated with Activin A and IL-6 could develop small-cell colonies (Figs. 4A, C, E and G), although the number of the colonies was smaller than that observed in the control. The cells surrounding the colonies

Fig. 2. Dose response of the effects of Activin A, IL-1 β , IL-6, and TGF- β on the DNA synthesis of hepatocytes in modified DMEM supplemented with 10 mM nicotinamide and 10 ng/ml EGF. Growth inhibitors were added to the culture medium at 48 h after plating. 40 μ M BrdU was added to the medium from 48 to 72 h. Cells were fixed with cold absolute ethanol at 72 h and immunocytochemistry for BrdU was carried out. Ten fields per dish and three dishes were counted at each point. The ordinate indicates the percent inhibition of DNA synthesis of cultured hepatocytes. The percentage was calculated using the labeling index of the control as 100%. The abscissa shows the concentration of each inhibitor. Each point shows the average and small bars show the SD.

Fig. 3. Immunocytochemistry for BrdU in primary culture of hepatocytes in modified DMEM supplemented with 10 mM nicotinamide and 10 ng/ml EGF (A). 10 ng/ml Activin A (B), 10 ng/ml IL-1 β (C), 10 ng/ml IL-6 (D), or 2 ng/ml TGF- β (E) was added to the culture medium from 96 to 144 h. 40 μ M BrdU was added from 96 to 144 h. The cells were fixed with cold absolute ethanol at 144 h and, thereafter, the immunocytochemistry for BrdU was carried out. Darkened nuclei are those stained with BrdU. Arrowheads show the small-cell colonies. All photos are the same magnification ($\times 118$).

showed little labeling, and empty areas were often observed in the cultures.

As we previously reported, the cells in focal colonies continued to proliferate with or without a growth fac-

tor (11). To clarify that the cells forming colonies without EGF were truly hepatocytes, immunocytochemistries for CK8 and Tf were carried out (Fig. 5) CK8 was positively stained not only in the cells of colonies but

Table I. EFFECTS OF MITO-INHIBITORY FACTORS ON THE FORMATION OF SMALL-CELL COLONIES IN PRIMARY CULTURES OF RAT HEPATOCYTES.

Inhibitor	No. of Experiments	Concentration of Inhibitor ng/ml	No. of Colonies %
Control	6	0	100 ^{a)}
Activin A	3	10	91.2 $\pm$ 4.8 ^{b)}
IL-1 β	6	10	63.2 $\pm$ 11.5
IL-6	6	10	89.1 $\pm$ 8.1
TGF- β	5	2	5.8 $\pm$ 2.8

Mito-inhibitory factors were added to the culture medium at 96 h after plating. Forty μ M BrdU was added from 96 to 144 h and the cells were fixed at 144 h.

^{a)} The number of colonies was calculated using that of the control as 100%. The number of colonies in the control was 17.7 $\pm$ 2.9 per 1,000 attached cells.

^{b)} The number shows the average $\pm$ SD.

Fig. 4. Immunocytochemistry for BrdU in primary culture of hepatocytes in modified DMEM supplemented with 10 mM nicotinamide and 10 ng/ml EGF. Ten ng/ml Activin A (A, E), 10 ng/ml IL-1 β (B, F), 10 ng/ml IL-6 (C, G), or 5 ng/ml TGF- β (D, H) was added to the culture medium at 48 h after plating. Some dishes (A, B, C, D) were washed twice with the culture medium without the inhibitors at 72 h and then maintained until 144 h. The others (E, F, G, H) were cultured with the inhibitors and the factors were added to the culture medium every 24 h. Forty μ M BrdU was added from 96 to 144 h. The cells were fixed at 144 h and the immunocytochemistry for BrdU was conducted. HE was used for counterstaining. Darkened nuclei are those stained with BrdU. Arrowheads show the small-cell colonies. Some cells treated with TGF- β have a large cytoplasm and multiple nuclei (arrows). The controls show features similar to the cells shown in Fig. 3A. All photos are the same magnification ($\times 118$).

also in the cells around colonies. However, non-parenchymal cells, which proliferated with time in culture, did not express this type of intermediate filament. Tf was well produced in the cells of colonies compared to the surrounding relatively large hepatocytes. In addition, as shown in Fig. 5C, Tf-R was remarkably ex-

pressed in the small cells. It seems that without a growth factor the cells in a focal colony are truly hepatocytes and may have high proliferative activity.

We examined whether the proliferation of the cells in the established colonies was regulated by mito-inhibitory factors. As shown in Fig. 6A, the number of the

Table II. EFFECTS OF TRANSIENT AND CONTINUOUS ADMINSTRATIONS OF MITO-INHIBITORY FACTORS ON THE FORMATION OF SMALL-CELL COLONIES IN PRIMARY CULTURES OF RAT HEPATOCYTES.

Treatment	Concentration ng/ml	No. of Colonies/1,000 cells	
		Periods of Treatment	
		48–72 h	48–144 h
Control	0	14.38 $\pm$ 2.40 ^{a)}	16.81 $\pm$ 1.50
Activin A	10	10.49 $\pm$ 1.14	3.47 $\pm$ 1.77
IL-1 β	10	5.56 $\pm$ 1.02	0
IL-6	10	11.95 $\pm$ 0.98	4.51 $\pm$ 1.14
TGF- β	2	0	0

The cells were treated with mito-inhibitory factors either from 48 to 72 h or from 48 to 144 h. Forty μ M BrdU was added to the culture medium from 96 to 144 h. The cells were fixed with cold absolute ethanol at 144 h.

^{a)} The number shows the average $\pm$ SD of three dishes.

Fig. 5. Immunocytochemistry for CK8, Tf, and Tf-R in primary culture of hepatocytes in modified DMEM supplemented with 10 mM nicotinamide. Ten ng/ml of EGF was added to the culture medium from 2 to 3 hrs after plating to day 6 and, thereafter, the cells were cultured without a growth factor. The cells were fixed with cold absolute ethanol at day 9 (A) or day 10 (B, C). Immunocytochemistry for CK8 (A), Tf (B), and Tf-R (C) was conducted and counterstained with hematoxylin. Darkened cytoplasm is those stained with each antibody. Arrowheads surround the small-cell colonies. An arrow (A) shows a nonparenchymal cell which is not stained with an anti-CK8 antibody. All photos are the same magnification ($\times 235$).

Fig. 6. Immunocytochemistry for BrdU in primary culture of hepatocytes in modified DMEM supplemented with 10 mM nicotinamide and 10 ng/ml EGF (A). Ten ng/ml Activin A (B), 10 ng/ml IL-1 β (C), 10 ng/ml IL-6 (D), or 2 ng/ml TGF- β (E) was added to the culture medium from day 7 to day 9. Forty μ M BrdU was added to the medium at day 8. The cells were fixed at day 9 and immunocytochemistry for BrdU was carried out. Darkened nuclei are those stained with BrdU. Arrowheads show the small-cell colonies. All photos are the same magnification ($\times 118$).

Table III. EFFECTS OF MITO-INHIBITORY FACTORS ON THE SMALL CELLS IN THE FOCAL COLONIES IN PRIMARY CULTURES OF RAT HEPATOCYTES.

Treatment	Concentration ng/ml	No. of Colonies	
		96–144 h ^{a)}	Day 8–Day 9 ^{b)}
Control	0	19.8±1.6 ^{c)}	12.2±1.9
Activin A	10	17.7±1.1	14.6±3.6
IL-1 β	10	14.7±1.7	13.6±4.5
IL-6	10	17.4±1.8	10.8±4.7
TGF- β	2	1.9±0.5	1.5±0.5

These results were obtained from experiments.

^{a)} Mito-inhibitory factors were added to the culture medium at 96 h and the cells were fixed at 144 h after plating. Forty μ M BrdU was added from 96 to 144 h.

^{b)} Mito-inhibitory factors were added to the medium at day 7 and the cells were fixed at day 9. Forty μ M BrdU was added from day 8 to day 9.

^{c)} The number shows the average±SD of three dishes.

cells in focal colonies increased compared to that of the cells in the colonies observed at 144 h. Some relatively large hepatocytes also synthesized their DNA even at day 9. Only TGF- β completely inhibited DNA synthesis of cells in focal colonies (Fig. 6E). Neither Activin A, IL-1 β , nor IL-6 could inhibit the BrdU-labeling of the cells both in and surrounding the colonies (Figs. 6B, C, and D). Table III shows the number of small-cell colonies at day 9 compared to the number observed at 144 h. The numbers of the colonies in the cultures treated with Activin A, IL-1 β and IL-6 were not significantly different from that of the control colony. TGF- β completely suppressed the number of colonies at day 9. Although the number of colonies at day 9 decreased in the culture treated with IL-1 β , IL-6 and Activin A compared to that of the colonies at 144 h, we must emphasize that each colony consisted of many small cells and became larger, and, therefore, it appeared that several small colonies fused and that these colonies looked like one large colony. Although the formation of the small-cell colonies was dependent on the growth factors (11), the proliferation of small cells was observed in the cultures without EGF after day 6. The growth of the small cells cultured without EGF was also inhibited only by TGF- β (data not shown).

DISCUSSION

In the present experiments we examined the effects of Activin A, IL-1 β , IL-6 and TGF- β on the formation of the small-cell colonies. As described in the Introduction, we have reported that rat hepatocytes are divided into three groups with regard to their capacity for proliferation (11, 12). Type I cells possess the highest potential to proliferate and can form colonies 4 to 5 days after plating. Most other hepatocytes in the adult rat seem to be Type II cells, for which the number of possible cell divisions is limited. As the DNA synthesis of most hepatocytes

in this culture condition started from 48 h after plating (data not shown), the initial 24-h treatment (48–72 h) with mito-inhibitory factors caused the suppression of the proliferation of many cells. As shown in Table II, transient treatment with TGF- β completely inhibited the formation of the colonies and the number of colonies observed at 144 h was reduced by half as a result of the transient treatment with IL- β . This result means that many Type I and Type II cells began their proliferation after 48 h culture and that TGF- β could permanently suppress the growth of both types of cells. On the other hand, IL-1 β could inhibit the DNA synthesis of both Type I and Type II cells but the effect was reversible. Therefore, the beginning of proliferation of Type I cells was delayed and the number of colonies observed at 144 h decreased. On the other hand, alteration of the sensitivity to IL-1 β was shown in the small cells of focal colonies between 96 h and day 8. Although the DNA synthesis of the small cells was partially inhibited at 96 h, the cells were resistant to IL-1 β at day 8. The different effects of IL-1 β and TGF- β on the inhibition of DNA synthesis in primary hepatocytes have been reported (14). The inhibitory effect of TGF- β was observed after treatment for only one hour in the early G1 phase while IL-1 β should act during the entire G1 phase in order to suppress the DNA synthesis of the cells. Although DNA synthesis of many hepatocytes was inhibited by Activin A and IL-6 as shown in Fig. 1, their transient additions did not interfere with the formation of the colonies. In addition, the continuous treatment did not completely suppress colony formation. Considering that the colony formation is dependent on the cell density, this result suggests that both Activin A and IL-6 have the ability to inhibit the proliferation of Type II cells but that they can not inhibit the DNA synthesis of Type I cells.

As is well known, each inhibitor has a specific receptor on the cell membrane. Hepatocytes express three

subtypes of TGF- β receptors (6, 28) and two types of Activin receptors (6). Although Activin is a member of the TGF- β gene superfamily and both receptors of TGF- β and Activin have serine/threonine kinase in their cytoplasmic domains (5, 22), TGF- β was reported not to bind Activin receptors (24). However, Type I cells may not express the Activin receptor on their cell membrane because Activin A can not inhibit the formation of the colony and the small cells in focal colonies. Moreover, Activin A can reversibly suppress the DNA synthesis of Type II cells while TGF- β irreversibly inhibited the proliferation of all types of hepatocytes. Finally, many cells treated with TGF- β were shown no be dead. The inhibitory mechanisms of Activin A and TGF- β in the DNA synthesis of rat hepatocytes may be different even if their receptors are members of the same family. On the other hand, the IL-6 receptor has been reported to be expressed in hepatocytes (17) and to be a member of both the immunoglobulin superfamily and the cytokine receptor family (23). Recently, a specific receptor of IL-1 β on rat hepatocytes was found by Kohira *et al.* (3), although the IL-1 receptor has been identified in other cells as well as hepatocytes (20). The latter receptor for IL-1 is a member of the immunoglobulin superfamily (6). The receptors of IL-1 β and IL-6 were shown to be members of the immunoglobulin superfamily, but the inhibitory mechanisms in the DNA synthesis of hepatocytes might be different because IL-6 did not have an inhibitory effect on Type I cells while IL-1 β could transiently inhibit the DNA synthesis of both Type I and Type II cells. In addition, the sensitivity of the small cells in focal colonies to IL-1 β changed during the proliferation of the colonies. These results suggest that Type I cells do not possess the IL-6 receptor and that the expression of the IL-1 β receptor on their cell membrane may change during the development of the small-cell colonies. Although we do not have any results on these receptors of rat hepatocytes, the present experiments suggest that the analyses of receptors may reveal the cell lineage from Type I cells to Type II cells and the role of these cells in the liver.

The development of small-cell colonies was shown in *in vitro* experiments using rat hepatocytes. It has been reported that small hepatocytes appear in a certain human liver disease (2, 21). Liver biopsy specimens from patients who suffered acute viral hepatitis and recovered from fulminant hepatitis, showed relatively small polygonal hepatocytes with small nuclei arranged in clusters in periportal areas. In addition, we pathologists often observe small cell clusters in liver specimens from long-term chronic hepatitis and liver cirrhosis. These small cells are generally understood as the regeneration of mature hepatocytes, but there is a possibility that hepatic progenitor cells like Type I cells may proliferate after the mature hepatocytes like Type II cells have been una-

ble to respond to growth stimulation. Although further experiments are necessary to confirm the existence of hepatic progenitor cells *in vivo*, our present results may give a clue to clarify the mechanisms of liver diseases.

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