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A Highly Efficient Method for Obtaining Clonal Epithelial Cells Proliferating in Cultures from Adult Rat Liver

— Development of Epithelial Cell Colonies in Cultures of Cells Derived from Nonparenchymal Liver Tissues —

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SUMMARY

Adult rat hepatocytes and nonparenchymal cells were dissociated by a collagenase-dispase perfusion method from livers of male Fischer 344 rats weighing 250 to 300 g. A hepatocyte-rich fraction (fraction I) and a fraction enriched with nonparenchymal cells (fraction II) were prepared by combing the liver followed by differential centrifugation. Another fraction rich in nonparenchymal cells (fraction III) was obtained from the residual tissues by dispase-digestion. Only a few colonies of epithelial cells per dish were obtained from fraction I which contained the greatest number of hepatocytes, whereas the greatest number of the colonies per dish were yielded from fraction II which contained the greatest number of nonparenchymal cells. When nonparenchymal cells of fraction III were seeded at the same density as that of fraction II, a much larger number of epithelial cell colonies were derived from the former than the latter. The residual tissues mainly consisted of portal tracts, accompanied by a few fragments of parenchymal tissues containing intralobular bile ductules and hepatic plates in periportal areas. When these fragments were excluded by a strictly performed combing procedure, fraction III was found to contain only a few or no hepatocytes, but epithelial cells were still derived from this fraction. These results suggest that adult rat liver epithelial cells proliferating in culture do not originate from hepatocytes, but from nonparenchymal cells which are located in periportal areas and in portal tracts.

Key words: Adult rat liver epithelial cells, Primary cultures

INTRODUCTION

A number of studies using adult rat liver epithelial cell lines or strains have revealed their usefulness for detection of carcinogenicity, mutagenicity and tumor-promoting activity of xenobiotics (12, 13, 16--22). On the other hand, many liver epithelial cell lines or strains have been reported to have some properties characteristic of hepatocytes (1, 2, 4, 7, 14). Recently, we have developed a highly efficient method for obtaining a large number of clonal proliferating epithelial cells from adult rat liver, leading to a reproducible establishment of liver epithelial cell strains (3). In addition, when cells dispersed by combing the liver were fractionated by differential centrifugation, a much larger number of epithelial cell colonies were found to be obtained from a nonparenchymal cell-rich fraction than from a hepatocyte-rich fraction (3). It is suggested that the liver epithelial cells proliferating in culture originate from cells other than hepatocytes.

Grisham (6) proposed that terminal bile ductular cells were "facultive stem cells" of liver epithelial cells propagable in culture. On the other hand, Yaswen *et al.* (23) obtained a population enriched with bile ductular cells from residual tissues following combing the liver perfused with collagenase. The purposes of this study are to determine whether epithelial cell colonies are derived from a nonparenchymal cell-rich fraction of residual tissues of the liver perfused with collagenase and dispase, and to compare the frequency of the colonies of this fraction with that of a nonparenchymal cell-rich fraction prepared by combing the liver.

MATERIALS AND METHODS

Animals. Male Fisher 344 rats weighing 250 to 300 g were used. The animals were maintained in a conventional 12-h light cycle room and given water and commercial pellet diet (MF, Oriental Yeast Co., Tokyo) *ad libitum* until the day of liver perfusion.

Cell Isolation. The procedure of liver perfusion with collagenase (Wako Chem. Co., Tokyo) and dispase II (Godo Shusei Co., Tokyo) was the cell isolation method II in our previous study (3) with some modifications. In the present study, the concentration of collagenase was increased to 120 U/m*l*. All solutions used for cell isolation contained 40 U/m*l* penicillin G and 40 μg/m*l* streptomycin sulfate instead of gentamicin sulfate. After the perfusion, the liver was combed in 600 pronase units (PU)/m*l* dispase II solution. When the cells were dispersed in the combing solution, the solution partly gelled. In order to prevent the solution from gelling, the solution containing cells was immediately diluted with an equal volume of Williams' medium E (WME) (Flow Lab., Irvine, UK) and pipetted repeatedly.

Cells dissociated by combing were fractionated according to the method of

differential centrifugation (3). Briefly, the isolated cell suspension rich in hepatocytes was obtained as the sediment by centrifugation at 50×g for 1 min, and designated as fraction I. The fraction enriched with nonparenchymal cells (NPC) was prepared as the sediment by a second centrifugation at 50×g for 5 min from the supernatant obtained at 50×g for 1 min. This fraction was designated as fraction II. Cells of each fraction were finally suspended in WME supplemented with 10% heat-inactivated calf serum (Grand Island Biol. Co., Grand Island, USA) (WME: $HICS_{10}$) and filtered through nylon mesh of 250 μm pore size.

Residual tissues following combing the liver were washed with WME and then suspended in 600 PU/m*l* dispase II in WME. The tissues were minced with fine scissors and incubated in this enzyme solution at 37℃ for 30 min. Following filtration through gauze, dissociated cells were washed three times by centrifugation at 50×g for 5 min and resuspension in WME: $HICS_{10}$. Isolated cells in the sediment were finally suspended in WME: $HICS_{10}$ and filtered through nylon mesh of 250 μm pore size. This cell population derived from the residual tissues was designated as fraction III.

The number of viable hepatocytes and "small cells" was determined using trypan blue dye exclusion test. The "small cells" were round-shaped NPC with a size similar to that of adult rat liver epithelial cells (ARL301-3 strain) which were detached from culture dishes. ARL301-3 strain is a non-transformed clonal strain established in our laboratory.

Culture Conditions. Cells of each fraction were seeded in 60×15 mm tissue culture dishes (Corning Glass Works, Corning, USA). One hour after seeding, the cells were washed twice with the culture medium consisting of WME supplemented with 10% heat-inactivated fetal bovine serum (Boehringer Mannheim GmbH, West Germany), 10^{-6} M dexamethasone (Sigma Chem. Co., St. Louis, USA), 40 U/m*l* penicillin G and 40 μg/m*l* streptomycin sulfate. The cells were maintained at 37.0℃ in a CO_2-incubator. The medium was changed on days 1, 4 and 7 after seeding, followed by twice a week feeding.

Colony Counting. Number of colonies per dish was determined at days 7 and 14 of culture. Following washing the cultures with WME, the cells were fixed for 5 min in methanol and then dried with cool blowing. The cells were stained for 3 min with 0.02% Coomassie brilliant blue dissolved in a mixture of acetic acid, methanol and distilled water. After washing with the solution without the dye, the cells were dried and counterstained with Giemsa. Coomassie blue staining method has been shown to visualize stress fiber of fibroblasts (11) and is useful for distinguishing morphologically rat liver epithelial cells from hepatocytes and other cells in culture (Y. Mochizuki and K. Furukawa, in preparation).

In the present study, the colonies were defined as those with an average of 10 or

more cells determined by the formula as follows: (Numger of cells on the longest axis of the colony+number of cells on the shortest axis of the same colony)/2.

Histochemical Examinations. The residual tissues following combing were fixed in ice-cold 5% (vol/vol) formalin in ethanol and embedded in soft-paraffin. The hisochemistry of γ-glutamyl transpeptidase (GGT) was made on deparaffinized sections according to the method of Kalengayi *et al.* (9), and on cultured cells as described previously (16).

RESULTS

Structural Characteristics of Residual Tissues Following Combing. Residual tissuen after combing were observed to consist of undissociated fibrous tissues containing a small amount of parenchymal tissues. Histologically, many ductal structures accompanied by arterioles, venules and lymph vessels were found in thick connective tissues (Fig. 1A). GGT activity was detected histochemically in the cells forming these ducts (Fig. 1B), indicating that these cells were interlobular bile ductal cells located in connective tissues of portal triads. A few GGT-positive

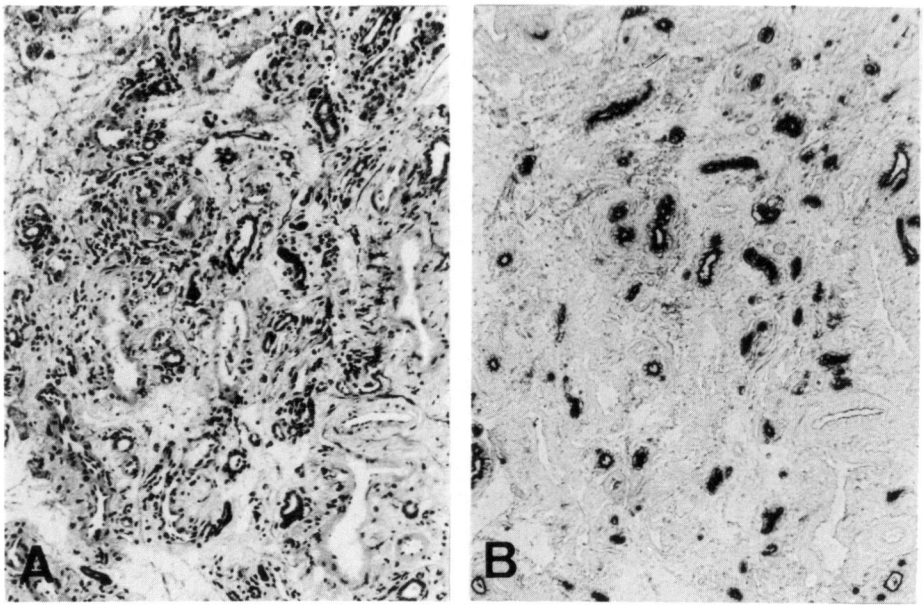

Fig. 1 Histology of residual tissues following combing of a male F344 rat liver perfused with collagenase and dispase II, ×45. *A*, Hematoxylin and eosin staining. Many interlobular ducts which are accompanied by portal venules, hepatic arterioles and lymph vessels, and surrounded by connective tissues are seen. *B*, GGT reaction of the serial section corresponding to that shown in *A*. Interlobular ductal cells are positive in GGT activity.

intralobular bile ductules and a few hepatocytes were also found in the residual tissues.

Morphological Characteristics of Proliferating Cells. Colonies of the two cell types were observed in primary cultures of all fractions. Epithelial cells (Fig. 2A) and fibroblast-like cells (Fig. 2B, C) derived from fraction III were the same in morphology as respective cells derived from fractions I and II. As shown in Fig. 2A, epithelial cells in the central portions of the colonies exhibited polygonal shapes and those located in the peripheral portions had slightly elongated shapes. Fibroblast-like cells in some portions of the colonies contained many stress fibers detected by Coomassie blue staining and showed epithelioid morphology (Fig. 2B). In other portions, fibroblast-like cells had a small amount of stress fibers and showed spindle shapes (Fig. 2C). From these chracteristic morphologies, epithelial cells were

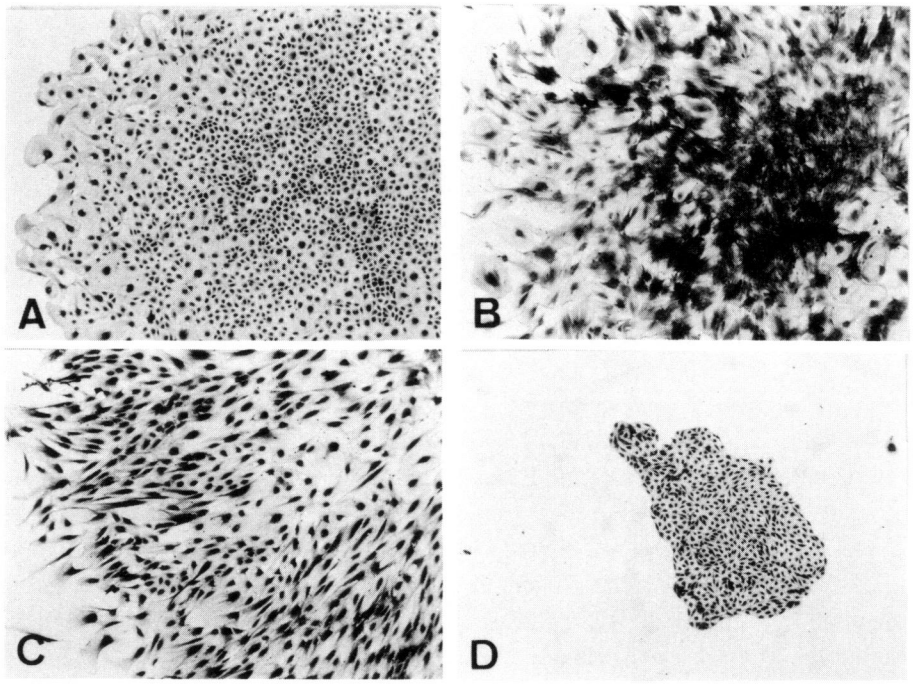

Fig. 2 Morphology of cells forming colonies in primary cultures at 14 days after culture. Coomassie brilliant blue and Giemsa staining, ×25. 6. *A*, Epithelial cells in a culture of fraction III. *B*, Fibroblast-like cells in a culture of fraction III. Many cells have a large amount of stress fibers. *C*, Fibroblast-like cells in other portions of the same colony as shown in *B*. The cells in this portion have a small amount of stress fibers. *D*, Endothelial-like cells in a culture of fraction III. They show a "cobblestone" appearance with a whirl-like arrangement in the colony, which has been reported to be characteristic of cultured endothelial cells (5, 8, 15).

Table 1 *Colony forming frequency of epithelial cells in primary cultures of cells in fractions prepared from adult rat liver.*

Fraction[a]		Seeding density per 60 mm dish[b]		Number of epithelial cell colonies per 60 mm dish[d]	
		Hepatocytes	Small cells[c]	Day 7	Day 14
I	*a*	1.50×10^6	1.48×10^5	2.8±1.0	3.2±1.3
	b	1.56×10^5	1.54×10^4	0	0
II	*a*	1.56×10^5	2.03×10^6	61±2	80±5
	b	4.11×10^3	5.34×10^4	1.7±0.8	1.4±0.5
III		6.60×10^3	5.34×10^4	47±2	43±3

[a] Cells isolated from a male F344 rat liver were fractionated as described in MATERIALS AND METHODS. Fraction I was enriched with hepatocytes and fraction II was enriched with nonparenchymal "small cells". These fractions were prepared by differential centrifugation from cells dissociated by combing, and fraction III was prepared from residual tissues following combing.

[b] Fractions I-*b* and II-*b* were prepared by dilution of fractions I-*a* and II-*a*, respectively, with WME : $HICS_{10}$. The seeding density of hepatocytes of fraction I-*b* is the same as that of fraction II-*a*. The seeding density of "small cells" of fraction II-*b* is the same as that of fraction III.

[c] The "small cells" were defined as those which were smaller in size than hepatocytes but similar to that of ARL301-3 cells as determined on hemocytometers.

[d] Values are means±SD and derived from 5 dishes of fraction I or II and 3 dishes of fraction III.

clearly distinguished from fibroblast-like cells.

In addition to the above mentioned two types of the cells, the third type cells were found in cultures of fraction III (Fig. 2D). They were smaller in size than the epithelial cells shown in Fig. 2A, and exhibited an epithelial morphology with a "cobblestone" appearance and a whirl-like arrangement, which have been reported to be characteristic of endothelial cells (5, 8, 15). These cells were therefore designated as "endothelial-like cells". Clonal growth of the three cell types were initiated on 3 to 6 days of primary culture.

Colony Forming Frequency at Day 7 and 14 of Culture. The number of epithelial cell colonies per dish of each fraction is shown in Table 1. A few colonies of epithelial cells were found in culutres of fraction I-*a* which contained the greatest number of hepatocytes. Fraction II-*a* contained the same number of hepatocytes as that of fraction I-*b* and the largest number of "small cells". From this fraction II-*a*, the largest number of epithelial cell colonies per dish were obtained, while no epithelial cell colonies were found in cultures of fraction I-*b*. Fraction III contained the same number of "small cells" as that of fraction II-*b*. However, a much

Table 2 *Colony forming frequency in primary cultures of cells in fractions prepared from adult rat liver : Experiment 1.*

Fraction[a]	I	II	III
Yield per liver :			
Hepatocytes	3.13×10^8	5.33×10^5	4.44×10^4
Small cells[b]	4.56×10^6	2.02×10^7	3.12×10^5
Seeding density per 60 mm dish :			
Hepatocytes	5.00×10^5	1.33×10^4	6.33×10^3
Small cells	4.38×10^3	5.00×10^5	4.44×10^4
Number of colonies per 60 mm dish[c] :			
Epithelial cells			
Day 7	0	12±3	5.3±0.6
Day 14	0.2±0.5	28±9	7.0±2.7
Fibroblast-like cells			
Day 7	0	2.8±8.9	7.3±0.6
Day 14	1.0±0.7	2.2±1.5	14±2
Endothelial-like cells			
Day 7	0	0	7.7±0.6
Day 14	0	0	6.0±3.6

[a] Cells isolated from a male F344 rat liver were fractionated as described in MATERIALS AND METHODS. Fraction I was enriched with hepatocytes and fraction II was enriched with " small cells ". These fractions were prapared by differential centrifugation from cells dispersed by a strictly performed combing, and fraction III was prepared from the residual nonparenchymal tissues.

[b] The " small cells " were difined as those which were smaller in size than hepatocytes but similar to that of ARL301-3 cells as deterimined on hemocytometers.

[c] Values are means±SD and derived from 5 dishes of fraction I or II and 3 dishes of fraction III.

larger number of epithelial cell colonien per dish were yielded from fraction III than from fraction II-*b*. On the other hand, this fraction III was about 1.5 times greater in number of hepatocytes than fraction II-*b*. It is indicated that an excess amount of undissociated parenchymal tissue fragments might remain with nonparenchymal fibrous tissues, and also that a large number of the cells which could proliferate in culture as epithelial cells might be located in the residual fibrous tissues and/or in the parenchymal tissue fragments.

To determine whether residual parenchymal tissues contain cells capable of proliferating in culture as epithelial cells, the liver was combed strictly and the residual tissues were washed completely with WME. In the two experiments presented in Tables 2 and 3, fragments of parenchymal tissues macroscopically could not be detected in the residual tissues. A few or no hepatocytes were

Table 3 *Colony forming frequency in primary cultures of cells in fractions prepared from adult rat liver : Experiment 2.*

Fraction[a]	I	II	III
Yield per liver :			
Hepatocytes	2.80×10^8	8.89×10^5	0
Small cells[b]	1.78×10^6	1.31×10^7	3.12×10^5
Seeding density per 60 mm dish :			
Hepatocytes	5.00×10^5	3.40×10^4	0
Small cells	3.18×10^3	5.00×10^5	4.44×10^4
Number of colonies per 60 mm dish[c] :			
Epithelial cells			
Day 7	0	27±4	3.7±0.6
Day 14	0	43±8	4.0±1.0
Fibroblast-like cells			
Day 7	0	5.4±0.6	25±1
Day 14	1.0±0.7	7.4±3.0	26±1
Endothelial-like cells			
Day 7	0	0	3.7±2.1
Day 14	0	0	3.5±1.3

[a] Cells isolated from a male F344 rat liver were fractionated as described in MATERIALS AND METHODS. Fraction I was enriched with hepatocytes and fraction II was enriched with "small cells". These fractions were prepared by differential centrifugation from cells dispersed by a strictly performed combing, and fraction III was prepared from the residual nonparenchymal tissues.

[b] The "small cells" were defined as those which were smaller in size than hepatocytes but similar to that of ARL301-3 cells as determined on hemocytometers.

[c] Values are means± SD and derived from 5 dishes of fraction I or II and 3 dishes of fraction III.

contained in fraction III of Experiment 1 (Table 2) and that of Experiment 2 (Table 3), respectively. On the other hand, numerous hepatocytes and "small cells" were yielded in fractions I and II, respetively, in both experiments.

Hepatocytes of fraction I and "small cells" of fraction II in these two experiments were seeded at 5.00×10^5 cells/dish. In cultures of fraction I, epithelial cell colonies were observed only at day 14 in Experiment 2 (Table 3) at a very low frequency. The largest number of epithelial cell colonies per dish were derived from fraction II of both experiments. "Small cells" of fraction III were seeded at 4.44×10^4 cells/dish in the two experiments, because a much lower yield of "small cells" was found in this fraction than in fraction II. This seeding density was about one 11th of that of the fraction II. At day 14 of the culture, the number of epithelial cell colonies per dish derived from fraction III were about one 4th in Experiment 1

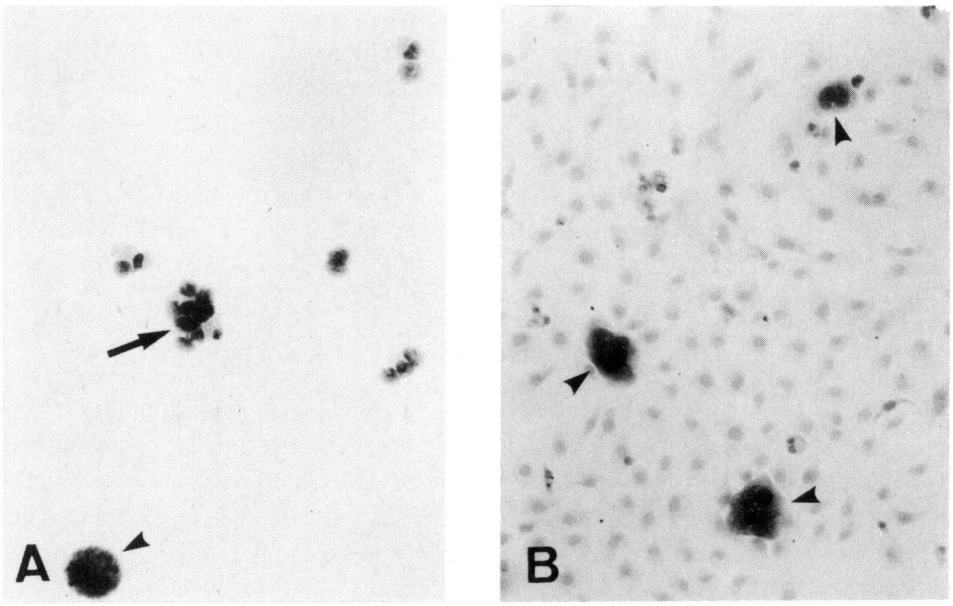

Fig. 3 GGT histochemistry of cells in primary cultures. GGT reaction counterstained with hematoxylin. *A*, Cells in a culture of fraction III at 1 hour after culture, × 280. Three darkly stained cells in the cluster of nonparenchymal cells (an arrow) are positive in GGT activity. The other cells including a hepatocyte indicated by an arrow head are GGT-negative. *B*, An epithelial cell colony in a culture of fraction III at 7 days after culture, ×112. Epithelial cells in the colony are negative in GGT activity, whereas hepatocytes (arrow heads) survived in the colony are GGT-positive.

(Table 2) and one 11th in Experiment 2 (Table 3) of that derived from fraction II of the respective enperiments. From the yields of hepatocytes and "small cells" per liver of each fraction and colony forming frequency of epithelial cells of each fraction in the two experiments, an average number of epithelial cell colonies per liver derived from fractions I, II and III at day 14 of culture were calculated to be 63, 1129 and 39, respectively.

A high colony forming frequency of fibroblast-like cells was detected in cultures of fraction III as shown in Tables 2 and 3. Colonies of endothelial-like cells were found exclusively in cultures of fraction III (Tables 2 and 3).

GGT Activity of Cultured Cells. After the washing at 1 hour of the culture, most of the NPC of fractions I and III did not show GGT activity when histochemically stained, and all NPC found in cultures of fraction II were negative in GGT activity. In cultures of fraction III, a few GGT-positive NPC were found in the NPC clusters, as shown in Fig. 3A.

On the 3rd to 6th day of culture when clonal growth of epithelial cells was initiated, GGT-positve NPC were still found in cultures of fractions I and III. On the other hand, GGT-positve epithelial cells were not observed. Through 14 days of culture, GGT activity was invariably absent in proliferating epithelial cells of all fractions (Fig. 3B).

Hepatocytes which survived up to 7 days exhibited a strong GGT staining in cultures of all fractions, as shown in Fig. 3B. In addition, GGT activity was faintly stained in fibroblast-like cells (not shown). Endothelial-like cells did not have a detectable activity of GGT.

DISCUSSION

The present study confirmed the high efficiency and reproducibility of the collagenase-dispase perfusion method (3) for obtaining propagable epithelial cells from adult rat liver. By this improvement of the cell isolation method, viable hepatocytes were yielded in fraction I at a level similar to that of the collagenase perfusion method reported previously (10), and a large number of NPC were yielded in fraction II. Moreover, cells in residual tissues following combing were also dissociated and collected as fraction III. The examinations of colony forming frequncy of epithelial cells of all fractions revealed that about 1.2×10^3 colonies could be obtained from an adult rat liver and the colonies were mainly derived from fraction II.

A comparative study on colony forming frequency of epithelial cells between fractions I and II has clearly revealed that a high frequency of the colonies is caused by high seeding density of " small cells " rather than hepatocytes. Moreover, as shown in Table 3, epithelial cell colonies were derived from fraction III containing no hepatocytes. These results strongly suggest that adult rat liver epithelial cells capable of proliferating in culture do not originate from hepafocytes, but from NPC.

The residual tissues were found to consist mainly of portal tracts which contained interlobular bile ductal cells showing GGT activity and endothelial cells, as nonparenchymal epithelial cell types. A few intralobular bile ductular cells which exhibited GGT activity and a few hepatoytes were also found, suggesting that periportal areas of lobules remained as fragments.

In the first examination of colony forming frequency(Table 1), the number of epithelial cell colonies per dish was much greater in cultures of fraction III than in those of fraction II when the seeding density of " small cells " in each fraction was adjusted to be the same. However, the other two examinations of fraction III obtained from the residual tissues following a strictly performed combing procedure revealed that the frequency relative to the seeding density of " small cells " in cultures of this fraction III was not different from that in cultures of fraction II.

These results suggest that many of the cells which might be progenitors of proliferating epithelial cells have been dissociated by a strictly performed combing procedure, and also that the residual fibrous tissues *per se* contain these progenitor cells. Therefore, cells capable of proliferating in culture as epithelial cells are most likely to be located in periportal areas and in portal tracts.

GGT activity was not detected in proliferating epithelial cells by the histochemical reaction. A few GGT-positive NPC were found in cultures of fractions I and III, while they were not found in cultures of fraction II from which the largest number of epithelial cell colonies were obtained. It is not clarified as to whether biliary epithelial cells are progenitors of epithelial cells proliferating in culture.

Appearance of GGT-positive cells following the exposure of adult rat liver epithelial cells to chemical carcinogens was reported (13). Moreover, ultrastructural and histochemical investigations showed that tumors grown in syngeneic rats after implantation of the transformed adult rat liver epithelial cells were well differentiated adenocarcinomas with high GGT activity (unpublished). Adult rat liver epithelial cells proliferated in culture could acquire GGT activity when they were transformed.

The present study showed that colonies of endothelial-like cells having a morpholoby distinctly different from that of epithelial cells were derived from fraction III. Adult rat liver epithelial cells capable of proliferation in culture are suggested not to originate from endothelial cells.

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