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A Comparison on Colony Formation of Primary Liver Culture between Adult and Aged Rats

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SUMMARY

The colony formation of primary liver culture of aged rats was compared with that of adult rats. The livers of adult (3 months old) and aged (2 years old) rat were perfused with collagenase and dispase, and the cells were dissociated by combing. Isolated cells were centrifuged at 50×g for 1 min. The sediments were rich in parenchymal hepatocytes (Fraction I). The supernatants were further centrifuged at 50×g for 5 min. The sediments were rich in non-parenchymal cells (Fraction II). The residual tissues after combing which were abundant in bile ductules were further digested in dispase, and the isolated cells were sedimented by centrifugation at 50×g for 5 min (Fraction III). The cultured cells were fixed in methanol on days 7 and 14, and stained with Coomassie blue and Giemsa. Three kinds of colonies, epithelial, endothelial, and fibroblastic, were counted in each fraction.

Epithelial colonies were most numerous in Fraction II, next in Fraction III and least in Fraction I of adult rats. Those of aged rats were found most in Fraction III, next in Fraction II and least in Fraction I. The total number of epithelial colonies per liver of aged rats was 1.6 times greater than that of adult rats. Although Fraction III of adult rats did not contain parenchymal hepatocytes in two rats, epithelial colonies were observed in mean numbers. These result strongly suggested that proliferative epithelial cells were derived from non-parenchymal cells. Furthermore, taking into account that the liver of aged rats contained many proliferative bile ductules and Fraction III of aged rats showed the most numerous epithelial colonies, proliferative epithelial cells might be derived from bile ductular cells.

Endothelial colonies were detected in Fraction III in both adult and aged rats. Since the residual tissues which were starting materials for Fraction III did not contain sinusoidal cells in general, proliferative endothelial cells were considered to have their origin in interlobular vessels.

Key words: Culture, Liver, Colony, Aged rat, Epithelial, Endothelial

INTRODUCTION

Procedures of primary hepatocyte culture have been established. However, the functions of hepatocytes were lost in a few days and parenchymal hepatocytes disappear within one or two weeks of culture. Instead of parenchymal hepatocytes, small, clear epithelial cells proliferate within several days of the culture. These epithelial cells can be propagable *in vitro,* and have been used as hepatocytes in various investigations including carcinogenesis. The origin of proliferative epithelial cells in culture is debatable. The liver contains several types of cells other than parenchymal hepatocytes, such as bile ductular and ductal cells, Kupffer cells, Itoh cells, endothelial cells, fibroblasts and so forth. Although propagable epithelial cells were considered to be derived from parenchymal hepatocytes because they retained some of hepatocyte properties(1-4, 6, 10, 15, 19, 21-23, 27, 29, 30, 31), they have been currently considered to be derived from non-parenchymal cells(14).

We recently developed a method for obtaining clonal epithelial cells proliferating in cultures from adult rat livers(8), implying that these cells originated from bile ductular cells. The livers of aged rats show a proliferation of bile ductules as well as enzyme-altered foci(24). If propagable epithelial cells are bile ductular cells in origin, epithelial colonies of primary liver cell culture of aged rats will be obtained in larger number than those of adult rats. This paper reports a comparison on colony formation of primary culture between adult and aged rat livers.

MATERIALS AND METHODS

Four male Fischer rats, each, 3 months old (adult) and 2 years old (aged), were used in the present study. The animals were maintained in special pathogen free room and given commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) and water freely.

Isolation and Fractionation of Cells. The procedures of cell isolation and fractionation were described previously(8). Briefly, the liver was perfused *in situ* through the portal vein, first with non-enzyme solution, second with collagenase solution (Wako Co., Tokyo, Japan), and then with dispase II solution (Godo Shusei Co., Tokyo, Japan). After the perfusion, the livers were combed in the dispase II solution. Cells dissociated by combing were suspended in Williams' medium E (WME) and centrifuged at 50×g for 1 min. The sediment was rich in parenchymal cells and designated as Fraction I. The supernatant was further centrifuged at 50×g for 5 min. The sediment was rich in non-parenchymal small cells and was termed as Fraction II. Residual tissue after combing, in which numerous bile ductules and ducts were present(8), were minced with fine scissors in dispase II solution and incubated at 37℃ for 30 min. The dissociated cells were suspended in WME and centrifuged three times at 50×g for 5 min. The sediment

was abundant in non-parenchymal small cells and was named Fraction III. All cells of the three fractions were resuspended in WME supplemented with 10% heat-inactivated calf serum (Grand Island Biol. Co., Grand Island, USA) and filtered through nylon mesh of 250 μm pore size. Cells were counted in a leukocyte counting chamber and the viability of parenchymal and non-parenchymal cells that exclude trypan blue was determined. Hepatocytes (HC) and non-parenchymal cells (NPC) were distinguished easily by their distinctive morphologic features, including size, shape and granularity of cytoplasm.

Culture Conditions. Cells of each fractions were seeded in 60×15 mm tissue culture dishes (Corning Glass Works, Corning, USA) at densities described in RESULTS, and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. One hour after seeding, cells were washed twice with the culture medium consisting of WME supplemented with 10% heat-inactivated fetal bovine serum (Boehringer Manheim 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 culture medium was changed on days 1, 4, 7, and 11 after seeding.

Colony Counting. The number of colonies per dish was calculated on days 7 and 14 of the culture. After washing the culture dishes with WME, the cells were fixed in methanol for 5 min and then dried with a cool blowing. The cells were stained for 3 min with 0.02% Coomassie brilliant blue(25). After washing with distilled water, the cells were counterstained with Giemsa. This staining method was quite useful to discriminate among parenchymal cells, epithelial cells, fibroblast, and other cells. In the present study, three kinds of colonies were detected with this staining; epithelial, fibroblastic, and endothelial. The colonies which consisted of 10 or more cells on the average of the number of cells on the long and short axes of colonies were counted as a colony(8, 9).

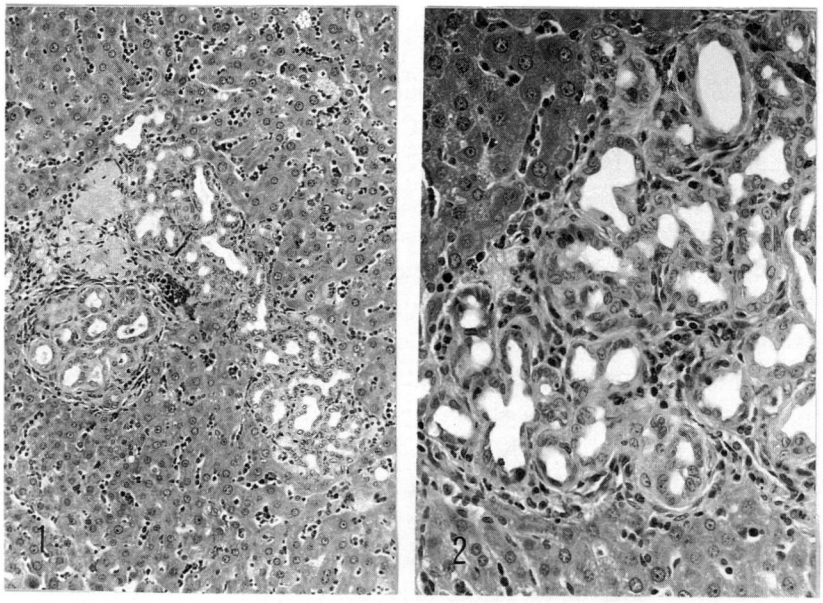
Histological Examination. The livers from adult and aged rats were fixed in buffered formalin, processed in the conventional method, and stained with hematoxylin and eosin.

Statistic Analysis. The results were subjected to statistic evaluation by means of the "t" test for small samples. Differences between means giving a probability of less than 5% were considered to be significant.

RESULTS

Histology of the Liver of Aged Rats

In the present study, 50 rats were kept for obtaining a sufficient number of aged rats with no tumors. Various tumors developed in these rats within 2 years. When any of the tumors became visible or when the rats were emaciated, they were killed and various organs were examined histologically. These rats were not used for cultures. Most of the livers had usually small lesions of proliferated bile ductules



Figs. 1 and 2 Histology of livers of aged rats. Note prominent proliferative bile ductules. Fig. 1, ×110, Fig. 2, ×220

(Fig. 1 and 2) and basophilic or clear foci of hepatocytes. Four rats which had no tumors except testiculars tumors were used in the present culture experiments.

Viability of Cells

Viability of isolated single cells and the yield of viable cells per liver were shown in Table 1. Viabilities of hepatocytes (HC) of Fraction I in both adult and aged

Table 1 *Yield of viable cells per liver and viability.*

	Adult		Aged	
	HC	NPC	HC	NPC
Fraction I	$2.66\times10^{8}\pm4.54\times10^{7}$ (81%)	$2.59\times10^{6}\pm1.05\times10^{6}$ (99%)	$2.19\times10^{8}\pm8.17\times10^{7}$ (84%)	$5.49\times10^{6}\pm2.17\times10^{6}$ (95%)
Fraction II	$1.13\times10^{6}\pm6.97\times10^{5}$ (38%)	$2.39\times10^{7}\pm1.01\times10^{7}$ (96%)	$1.57\times10^{6}\pm9.53\times10^{5}$ (43%)	$3.67\times10^{7}\pm1.11\times10^{7}$ (93%)
Fraction III	$1.33\times10^{5}\pm2.11\times10^{5}$ (81%)	$2.89\times10^{5}\pm2.57\times10^{4}$ (97%)	$5.00\times10^{5}\pm3.65\times10^{5}$ (74%)	$8.46\times10^{5}\pm4.98\times10^{5}$ (98%)

HC; hepatocyte, NPC; non-parenchymal cell.
Values are means±SD of four experiments.
Parentheses mearn viability.

rats were about 80%. However, those of Fraction II were around 40%, irrespective of being adult or aged rats. This result suggested that the supernatant of Fraction I contained many of dead hepatocytes. Non-parenchymal cells (NPC) in the three fractions showed higher viabilities than HC, irrespective of being adult or aged rats, implying that NPC cells are more resistant to collagenase and dispase II.

The viable parenchymal cells in Fractions I and II were not different in number between adult and aged rats, while NPC of aged rats in Fraction I and III were two and four times greater than those of adult rats, respectively. The ratios of HC and NPC varied in three fractions. Fraction I contained more HC than NPC and Fraction II had a large number of NPC. The viable cells in Fraction III were limited, and hepatocytes were not present in Fraction III of two adult rats. The seeding density of Fraction I was fixed at 5×10^5 cells as hepatocytes and that of Fraction II at 5×10^5 cells as NPC. These two fractions were seeded in ten dishes, and five dishes were examined on day 7 and other five dishes on day 14. The seeding density of Fraction III was 4.44×10^4 cells as NPC. Fraction III of aged rats was seeded in ten dishes, whereas that of adult rats was seeded in 5 to 7 dishes because of the pauctiy of cells in Fraction III of adult rats.

Morphology of Colonies

Representative photographs of Coomassie blue-Giemsa stained dishes were presented in Figs. 3 and 4. Three kinds of colonies were observed in the present study as reported previously(8); namly, epithelial, endothelial and fibroblastic. The epithelial colonies consisted of flattened round cells, measuring 50–100 μ in diameter (Figs. 5–8). The cells contained one or two nuclei which were round or ovoid in shape. The cytoplasms were very clear and contained a few organelles around the nuclei. The cells were arranged in pavement pattern. The cells situated in center of colonies were generally small in size and were stained more strongly. Mitoses were frequently observed.

The endothelial colonies consisted of smaller and fusiform cells than epithelial cells, measuring 60 μ in the long axis and 20 μ in the short axis (Figs. 9–10). The nuclei were also elipsoid in shape. The cytoplasms were less abundant than those of epithelial cells, but contained more abundant fibrils. Mitoses were hardly observed. The cells were contacted each other so intimately that the margins of colonies were very sharp.

The fibroblastic colonies consisted of the largest flattened cells among the three kinds of colonies (Figs. 11–12). Their shapes were irregular and were arranged randomly. The cytoplasms had abundant stress fibers. Mitoses were occasionally found.

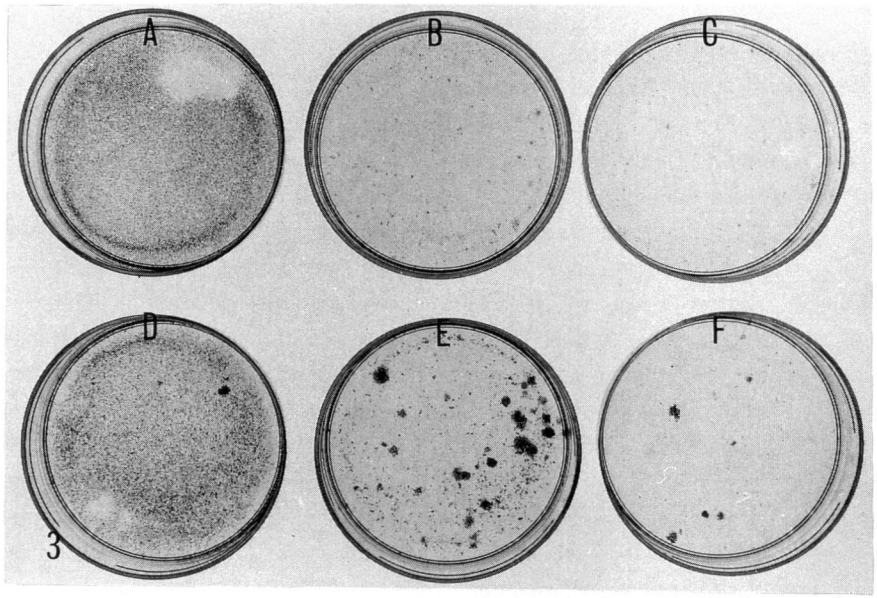


Fig. 3 Representative photographs stained with Coomassie blue and Giemsa of adult rats. A, B, and C are Fraction I, II, and III on Day 7, respectively. D, E, and F are Fraction I, II, and III on Day 14, respectively.

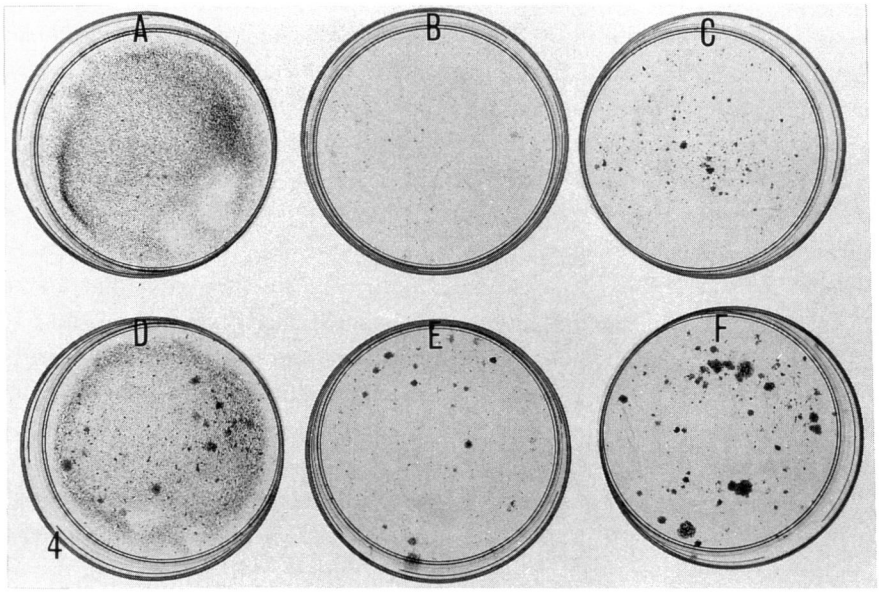
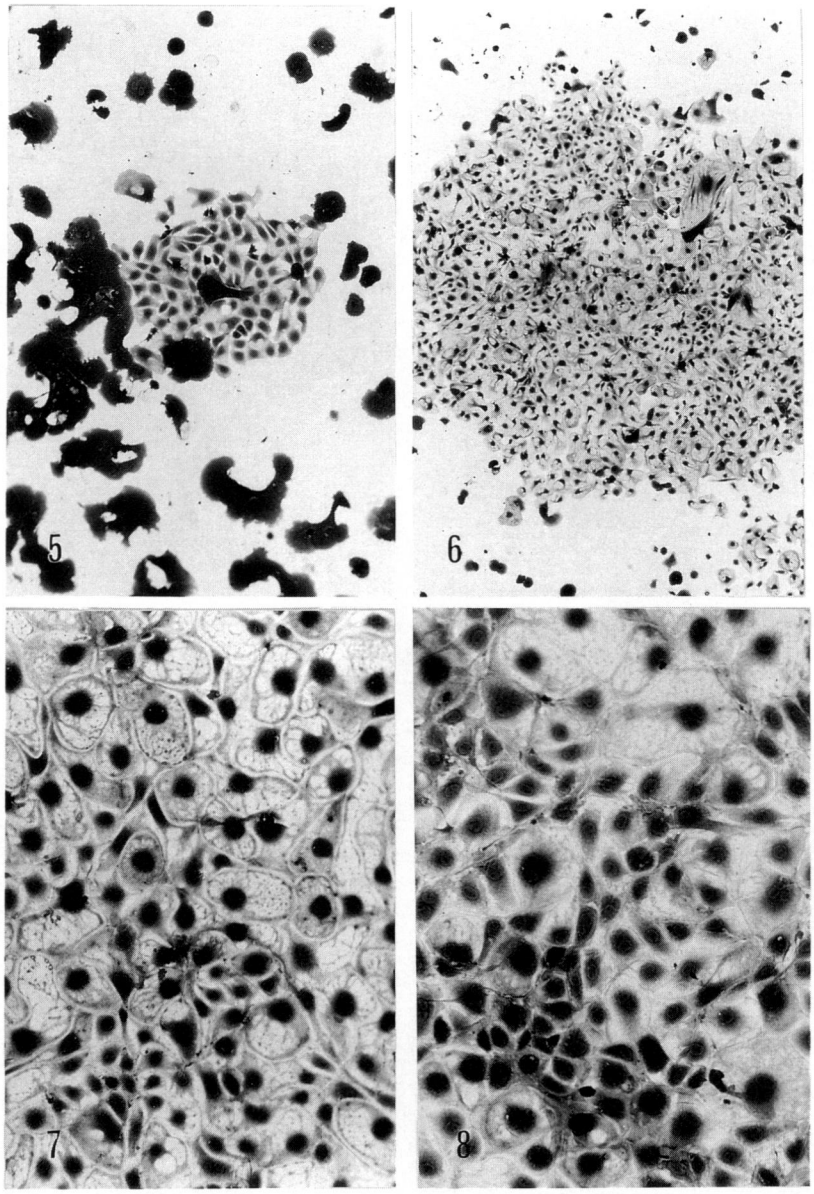
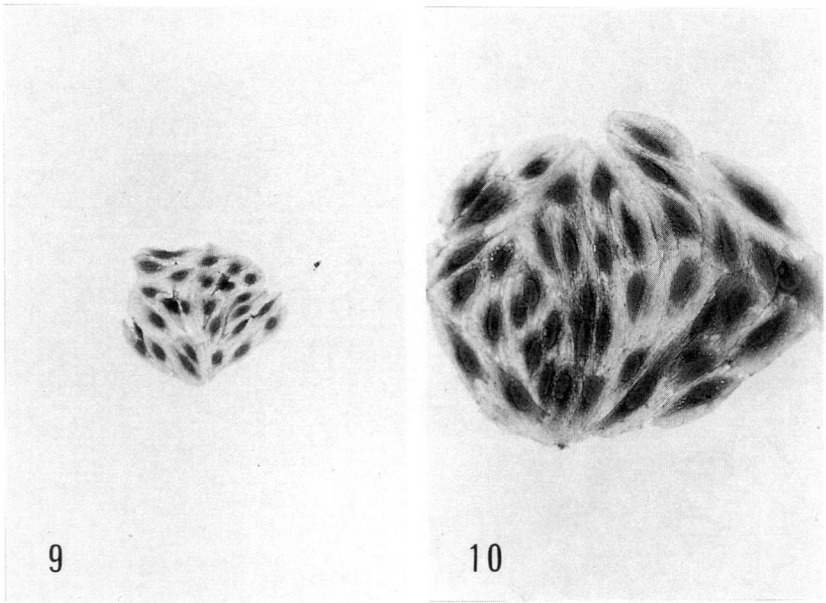


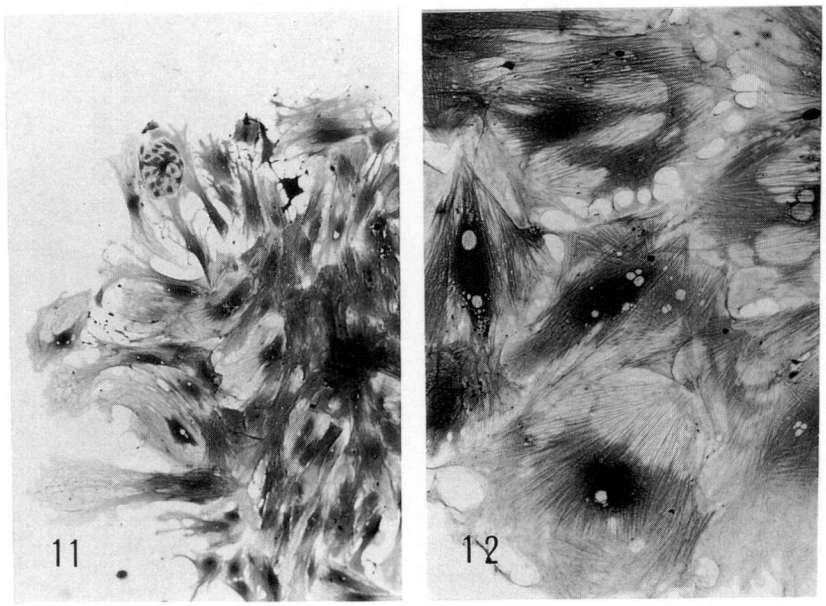
Fig. 4 Representative photographs stained with Coomassie blue and Giemsa of aged rats. A, B, and C are Fraction I, II, and III on Day 7, respectively. D, E, and F are Fraction I, II, and III on Day 14, respectively.



Figs. 5–8 Epithelial colonies stained with Coomassie blue and Giemsa. Fig. 5; Fraction I of aged rats on Day 7. ×55. Fig. 6; Fraction I of aged rats on Day 14. ×22. Fig. 7; Fraction II of adult rats on Day 14. ×83. Fig. 8; Fraction II of adult rats on Day 14. ×118.



Figs. 9–10 Endothelial colonies stained with Coomassie blue and Giemsa. Fig. 9; Fraction III of aged rats on Day 14. ×110. Fig. 10; Fraction III of aged rats on Day 14. ×220.



Figs. 11–12 Fibroblastic colonies stained with Coomassie blue and Giemsa. Fig. 11; Fraction III of aged rats on Day 14. ×55. Fig. 12; Fraction III of adult rats on Day 7. ×165.

Numbers of Colonies

The numbers of three kinds of colonies in each fraction were shown in Table 2. The epithelial colonies were hardly detected in Fraction I of adult rats. Those of Fraction I in aged rats were found with a frequency of 1 per dish on Day 7 and 3 per dish on Day 14. However, no significance could be obtained between adult and aged rats, or between Day 7 and 14. A dozen epithelial colonies per dish were detected in Fraction II of adult rats on Day 7 and two dozen or so epithelial colonies on Day 14. Those in aged rats were one third of those in adult rats. However, those of Fraction III in aged rats were five times more numerous than those in adult rats. These results suggested that the residual tissues after combing of aged rats contained more proliferative epithelial cells, while in adult rats most of these cells were obtained in Fraction II. The total numbers of epithelial colonies per liver on Day 14 derived from adult and aged rats were 1557 and 2512, respectively. Although larger epithelial colonies were observed in Day 14 than in Day 7, the significant differences in number between Day 7 and Day 14 were not obtained in any fractions. In Fraction III of two adult rats, hepatocytes were not obtained, whereas epithelial colonies were detected with a frequency of 4 per dish.

No endothelial colonies were observed in Fraction I. In Fraction II, only aged rats on Day 14 showed very few endothelial colonies. However, these colonies were observed considerably in Fraction III, irrespective of adult or aged rats. One aged rat showed an extremely high frequency of endothelial colonies in Fraction III, 32 per dish on Day 7 and 29 on Day 14. These values were not included in Table 2. These results suggested strongly that almost all proliferative endothelial cells remained in the residual tissues.

Fibroblastic colonies were detected in all fractions. Fraction I in both adult and aged rats on Day 7 showed the smallest number of colonies, and Fraction III in

Table 2 *Numbers of colonies per dish.*

		Fraction I		Fraction II		Fraction III	
		Adult	Aged	Adult	Aged	Adult	Aged
Epithelial	Day 7	0.05±0.09	1.10±0.70	13.25±9.73	5.00±2.28	3.70±1.65	15.25±2.50[a]
	Day 14	0.25±0.25	3.08±2.39	29.25±11.98	10.40±6.00	4.05±2.37[a]	21.00±4.55[a]
Endothelial	Day 7	0	0	0	0	4.40±3.01	4.93±2.72
	Day 14	0	0	0	0.10±0.20	2.63±2.69	4.73±1.67
Fibroblastic	Day 7	0.05±0.10	0.75±0.38[a]	2.55±2.11	0.65±0.41	17.10±9.00	2.60±1.03
	Day 14	2.80±2.46	2.85±0.98[b]	4.40±3.66	0.85±0.44	18.00±5.94	4.85±1.24[a,b]

Values are means±SD of four experiments.

a; significantly different from adult rats.

b; significantly different from Day 7.

adult rats showed the largest number of colonies. Significant differences between adult and aged rats were obtained in Fraction I on Day 7 and Fraction III on Day 14, and those between Day 7 and Day 14 in Fraction I and Fraction III of aged rats.

DISCUSSION

The present experiments clearly showed that epithelial colonies were more numerous in the aged rats than in adult rats. The total number of epithelial colonies in aged rats was 1.6 times greater than in adult rats. The yield of the epithelial colonies varied among three fractions. More numerous epithelial colonies were found in Fraction II and III than Fraction I in both adult and aged rats, implying that the proliferative epithelial cells were derived from NPC. The numbers of epithelial colonies in Fraction II in aged rats were about one third of those in aged rats, whereas those in Fraction III in aged rats were about five times greater than those in adult rats, suggesting that the proliferative epithelial cells in aged rats were dissociated by enzymes at a lesser rate than those in adult rats and consequently they remained in residual tissues. Taking into account the fact that seeding of Fraction III were about one eleventh of those of Fraction II as NPC, Fraction III contained huge numbers of proliferative epithelial cells, especially in the case of aged rats.

While in Fractions II and III, the yields of hepatocytes were much less than in Fraction I, epithelial colonies were more numerous in Fraction II and III than in Fraction I. Further more, in two adult rats, the yields of hepatocytes were nil, but the numbers of epithelial colonies per dish were the same as those in the other two adult rats. These results strongly suggested that the proliferative epithelial cells were derived from NPC rather than parenchymal hepatocytes.

Non-parenchymal cells of livers consisted of bile ductular and duct cells, Kupffer cells, Itoh cells (fat storing cells), endothelial cells, fibroblasts and so forth. Ultrastructural investigations of propagable epithelial cells revealed junctional complexes characteristic of epithelial cells(2, 4, 8, 10, 17, 18, 20, 23, 26, 28). These observations may deny that proliferative epithelial cells are Kupffer cells, Itoh cells and fibroblasts in origin. In the present study, the cells of fibroblastic colonies and endothelial colonies were distinct morphologically from the cells of epithelial colonies. Furthermore, proliferative epithelial cells did not show phagocytosis(9).

The propagable epithelial cells had some properties of parenchymal hepatocytes (1-4, 6, 10, 15, 19, 21-23, 27, 29, 30, 31). Therefore, these epithelial cells were considered to be derived from parenchymal hepatocytes by many investigators. Indeed, Williams *et al*(31) found peroxisomes typical of parenchymal hepatocytes in the cells of epithelial cell lines derived from 10 days-old rat, and Karasaki *et al* (18) found bile canalicular-like space in transformed liver epithelial cell lines.

However, Grisham *et al*(14) showed clearly that the proliferative epithelial cell colonies were derived from small cells different from parenchymal hepatocytes, and considered that these small cells were derived from facultative hepatic stem cells located in the terminal biliary ductules. The results of the present experiments support Grisham's hypothesis, because many epithelial colonies were detected in Fraction III which were rich in bile ductules(8) and had none or less hepatocytes. Moreover, the fact that Fraction III of aged rats which were obtained from residual tissues containing many proliferative bile ductules showed more numerous epithelial colonies than in adult rats suggests strongly that the proliferative epithelial cells are derived from bile ductule cells.

It should be mentioned that, in the present experiments, many endothelial colonies were detected in Fraction III. The residual tissues after combing which were the starting materials for Fraction III contained arterioles, venules and lymph vessels as well as bile ductal structures and connective tissues but did not contain the sinusoidal endothelial cells in general(8). The sinusoidal endothelial cells can be isolated and cultured until 24 hrs(5). Our endothelial colonies resembled much more those derived from bovine aorta(13), from adrenal capillaries of bovine, human and rats(7), from foreskin capillaries(7), from thoracic ducts in human and canine(12), and from human umbilical cord veins(11) than sinusoidal endothelial cells colonies. Although the endothelial cells of present experiments grow very slowly, the cloning of the cells is in progress.

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