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The origin of intrahepatic bile duct cells in the mouse

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SUMMARY

The origin of the intrahepatic bile ducts in the embryonic mouse liver was investigated. At 12·5 and 13·5 gestation days in the C3H/Tw strain mouse, the liver parenchyma contains morphologically and histochemically homogeneous immature hepatocytes but not bile duct cells. When the liver fragments were cultured in the testis, immature hepatocytes differentiated into large hepatocytes for the most part and also into bile duct cells. In contrast, when the similar liver fragments were cultured under the skin of newborn mice, bile duct cells differentiated much earlier in all transplants than those cultured in the testis. These bile duct cells were considered to be the intrahepatic bile duct cells, since they did not form biliary glands but possessed a basal lamina and histochemical characteristics of intrahepatic bile duct cells of the normal liver. The origin of the endodermal epithelial cells in the mouse liver is discussed with special attention to the differentiation of the intrahepatic bile duct cells from the immature hepatocytes.

INTRODUCTION

Endodermal components of the liver in the mouse are hepatocytes and bile duct cells. Both the cells develop in close vicinity to each other at the same time. The hepatic diverticulum of the mouse embryo consists of cranial and caudal portions. The endodermal cells in the cranial portion differentiate into hepatocytes, intrahepatic bile duct cells and hepatic duct cells, and the cells in the caudal portion differentiate into other extrahepatic bile duct cells (Shiojiri, 1979 and 1981). The origin of the intrahepatic bile duct cells in mammals has been controversial. Two main theories have been advocated. One is that the intrahepatic bile duct cells differentiate from hepatocytes (Lewis, 1911; Bloom, 1926; Horstmann, 1939; Elias, 1955; Du Bois, 1963; Wilson *et al.* 1963; Wood, 1965; Picardi *et al.* 1968*a* and *b*; Enzan *et al.* 1974), and the other is that the intrahepatic bile ducts originate from hepatic ducts (Hammar, 1926; Koga, 1971). I have shown enzymo- and immunohistochemically that the precursor cells of the intrahepatic bile duct cells are endodermal cells constituting lumen structures, and that they are intermediate in characteristics between immature

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hepatocytes and hepatic duct cells in the mouse embryo (Shiojiri, 1981).

Doljanski & Roulet (1934) showed that hepatocytes can construct a tube possessing a large lumen in a mixed culture with connective tissue. However, they did not demonstrate that the tube is the bile duct. The differentiation of bile duct cells from isolated mammalian hepatocytes transplanted *in vivo* has been also controversial (Jirtle *et al.* 1980; Ebata & Mito, 1981).

The present study will demonstrate that the mouse immature hepatocytes can differentiate not only into mature hepatocytes, but also into intrahepatic bile duct cells when embryonic liver fragments containing only immature hepatocytes as the component of the endodermal epithelia are cultured in the testis or under the skin.

MATERIALS AND METHODS

Animals

Embryos were obtained from C3H/Tw strain mice bred at the Zoological Institute of the University of Tokyo. The animals were mated during the night and copulation was confirmed by the presence of the vaginal plug the next morning. The conceptus was considered 0·5 days at twelve noon of this day.

Transplantation of liver fragments into the testis

To confirm the maintenance and differentiation of intrahepatic bile duct cells in the testis, 18·5-day embryonic liver fragments ($1 \times 1 \times 1\,mm^3$) (Fig. 1) containing intrahepatic bile duct cells were transplanted with carbon particles into the testis of the syngeneic mice with a glass capillary.

In this strain of mouse, the precursor cells of the intrahepatic bile duct cells begin to develop in the hilus at 14·5 days of gestation, and the liver parenchyma at 12·5 and 13·5 days contains no bile duct. Each lobe of the 12·5- and 13·5-day liver was cut into two parts: proximal and distal to the hilus (Fig. 1). These liver fragments and the remaining hilus parts containing the extrahepatic bile duct (Fig. 1) were transplanted into the testis. To ascertain whether bile duct cells can differentiate into hepatocytes, the common bile duct was also transplanted. The transplants were recovered after 1, 3 and 8 weeks.

Transplantation of liver fragments under the skin of newborn mice

Since it is in a foetal stage when intrahepatic bile duct cells differentiate, 13·5-day liver fragments containing only immature hepatocytes as an endodermal epithelium were cultured under the skin of newborn mice to show the rapid differentiation of intrahepatic bile duct cells from the immature hepatocytes in ectopic sites. Liver fragments (Fig. 1) were marked with carbon particles and transplanted under the ventral skin of newborn mice or into a fat pad of the mice with a fine forceps. The transplants were recovered after 1, 3 and 8 weeks.

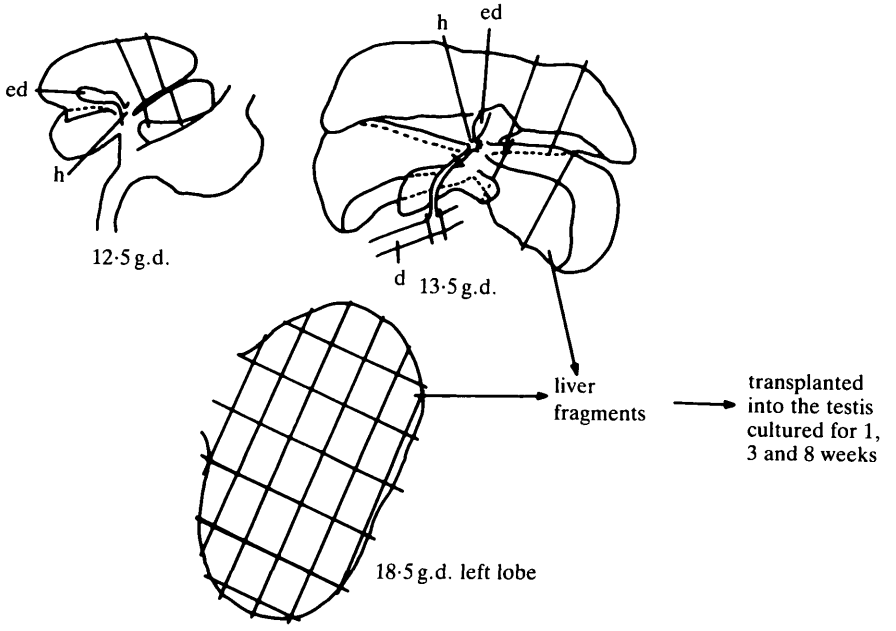


Fig. 1. Mode of dissection of 12·5-, 13·5- and 18·5-day livers. Livers of 12·5- and 13·5-day embryos are ventrally viewed, and the 18·5-day liver is cranially viewed. ed, extrahepatic bile duct. h, hilus. d, duodenum. g.d., gestation days.

Histological technique

The transplants were fixed in Bouin's fluid and embedded in paraffin. Serial sections at 6 μm for testicular transplants and at 7 μm for subcutaneous transplants were stained with periodic acid–Schiff–haematoxylin (PAS-HX).

The number of bile duct cells was counted in every other section, and the proportion of bile duct cells to the total endodermal epithelial cells was calculated in one transplant. The results were analysed statistically by the Student's t-test, and differences were considered significant when P was smaller than 0·05.

Histochemistry

To confirm the differentiation of mature hepatocytes and intrahepatic bile duct cells, cellular localization of albumin, alphafoetoprotein, alkaline phosphatase activity and glycogen in intratesticular transplants was analysed histochemically according to Horikawa *et al.* (1976) and Shiojiri (1981). Tissue pieces for albumin and alphafoetoprotein were fixed in a cold mixture of 96 % ethanol and glacial acetic acid (99: 1 v/v) for 20–24 h, and embedded in paraffin. Sections at 6 μm were cut. Localization of albumin and alphafoetoprotein in the transplants was examined by an indirect immunofluorescent method. Antisera used in this study (anti-mouse albumin antisera (rabbit), anti-mouse alphafoetoprotein antisera (rabbit) and fluorescein isothiocyanate-conjugated anti-rabbit IgG antibodies

(goat)) were purchased from Miles Laboratories (Indiana). The sections were also stained with haematoxylin and eosin (HX–E) after the immunofluorescence was examined.

Electron microscopy

Testicular transplants were fixed in a modified Karnovsky (1965)'s fixative (4% paraformaldehyde and 2·5% glutaraldehyde in 0·1 M-phosphate buffer, pH 7·4) for 2 h at 4 °C and postfixed in 1% osmium tetroxide buffered with 0·1 M-phosphate (pH 7·4) for 2 h at 4 °C. After dehydration in a series of ice-cold graded ethanol, the blocks were embedded in Epon (Luft, 1961). Sections were cut on a Porter-Blum MT2 microtome, stained with uranyl acetate and lead citrate, and examined with a JEOL-JEM 100CX electron microscope.

RESULTS

1. *Differentiation of intrahepatic bile duct cells in 18·5-day liver fragments cultured in the testis*

When 18·5-day liver fragments (at this stage, the proportion of fragments containing intrahepatic bile duct cells to the total fragments was 22/24) were cultured in the testis, the proportion of transplants containing bile duct cells to the total transplants decreased temporally (after 1 week, 13/22; after 3 weeks, 7/21), but bile duct cells were observed in all transplants after 8 weeks (15/15). These bile ducts differentiating in the testis were intrahepatic bile ducts in type, since they had no biliary glands characteristic of the extrahepatic bile duct including hepatic ducts. Histologically, the 18·5-day fragments in the testis developed sinusoids and mature hepatocytes, and the histology was similar to that of the normal adult liver (Fig. 2).

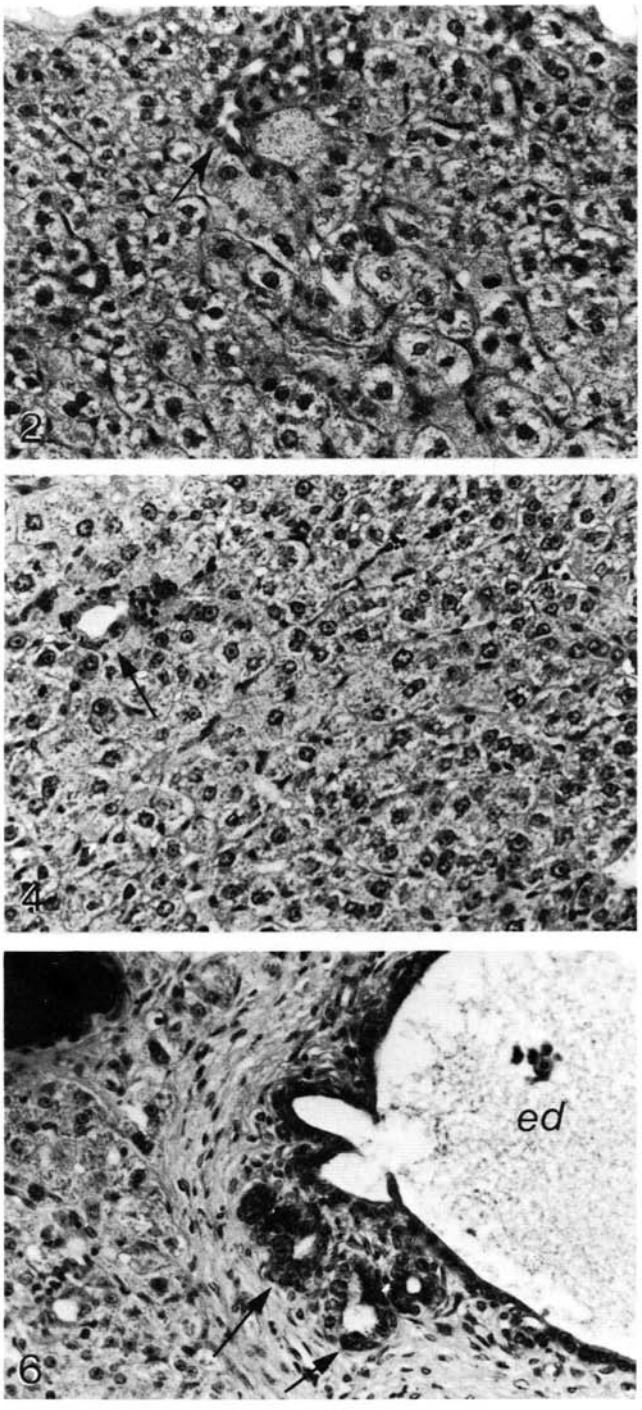
2. *Differentiation of bile duct cells in 12·5- and 13·5-day liver fragments cultured in the testis*

The results of differentiation of bile duct cells in 12·5- and 13·5-day liver fragments transplanted into the testis and cultured for 8 weeks are shown in Fig. 3.

Fig. 2. A section of a liver fragment of an 18·5-day embryo transplanted into the testis and fixed after 8 weeks. Arrow indicates intrahepatic bile duct cells. PAS-HX. ×250.

Fig. 4. A section of a liver fragment of a 13·5-day embryo transplanted into the testis and fixed after 8 weeks. Intrahepatic bile duct cells (arrow) differentiated. PAS-HX. ×250.

Fig. 6. A section of a 12·5-day liver fragment with extrahepatic bile duct (ed) transplanted into the testis and fixed after 8 weeks. Arrows indicate biliary glands. PAS-HX. ×250.



Figs 2, 4 & 6

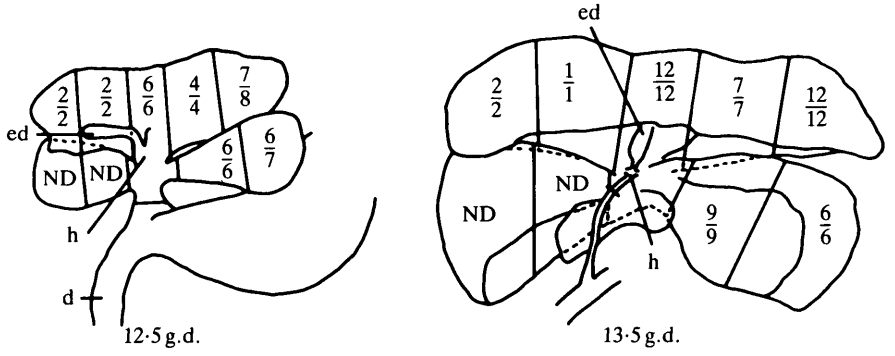


Fig. 3. Differentiation of bile duct cells from immature hepatocytes in 12·5- and 13·5-day liver fragments transplanted into the testis and cultured for 8 weeks. The ratio represents the number of transplants showing the bile duct cell differentiation to the total number of transplants. Livers are ventrally viewed. ed, extrahepatic bile duct. h, hilus. d, duodenum. g.d., gestation days. ND, not determined.

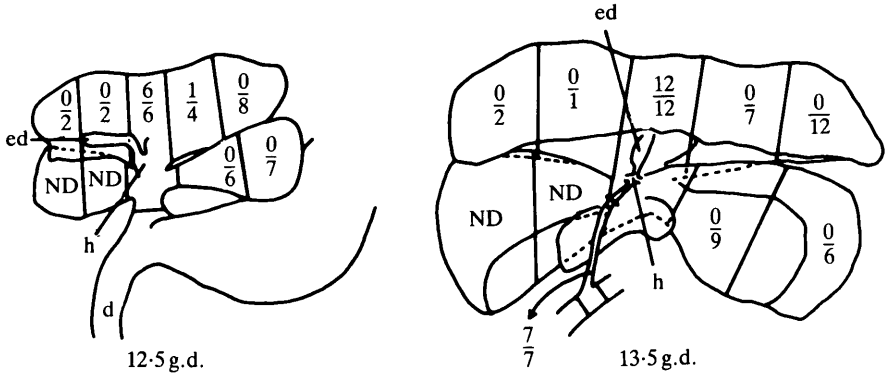


Fig. 5. Differentiation of biliary glands developed in 12·5- and 13·5-day liver fragments transplanted into the testis and cultured for 8 weeks. The ratio represents the number of transplants showing the biliary gland formation to the total number of transplants. Livers are ventrally viewed. ed, extrahepatic bile duct. h, hilus. d, duodenum. g.d., gestation days. ND, not determined.

Immature hepatocytes in the fragments differentiated into mature hepatocytes for the most part, and also differentiated into bile duct cells, independently of the lobes (Figs 3 and 4). In the left lobe of 13·5-day livers, the proportions of bile duct cells to the total endodermal cells in testicular transplants were $5{\cdot}5 \pm 3{\cdot}5$ % (five transplants) in the proximal portion and $6{\cdot}1 \pm 3{\cdot}1$ % (five transplants) in the distal portion. There was no significant difference in the proportion of bile duct cells to the total endodermal cells between both the portions ($P > 0{\cdot}05$). The bile ducts differentiated in liver fragments not containing extrahepatic bile duct cells at the transplantation were morphologically identified as intrahepatic bile duct cells (Fig. 5). Biliary glands were formed in all liver fragments containing the extrahepatic bile duct (Figs 5 and 6). The bile ducts differentiated in the testis

did not always connect with each other along the vessels, though the biliary system in the normal liver are organized along the portal vein like a branching tree. Free ducts, which were not connected with hepatocytes and other bile ducts, were also observed.

Fig. 7 shows the time course of the bile duct formation in the testis. Bile duct cells differentiated more rapidly in 13·5-day transplants than in 12·5-day liver transplants, and in the proximal portion than in the distal portion. The most rapid formation of bile ducts occurred in the liver fragments containing the extrahepatic bile duct. The bile duct formation occurred in the liver parenchyma close to the extrahepatic bile duct after 1 and 3 weeks' cultivation, but it occurred throughout the liver parenchyma after 8 weeks' cultivation.

Liver fragments from 12·5- and 13·5-day embryos transplanted into the testis and cultured for 8 weeks were also rich in sinusoids, and showed a histology similar to that of the adult liver as well as 18·5-day liver fragments.

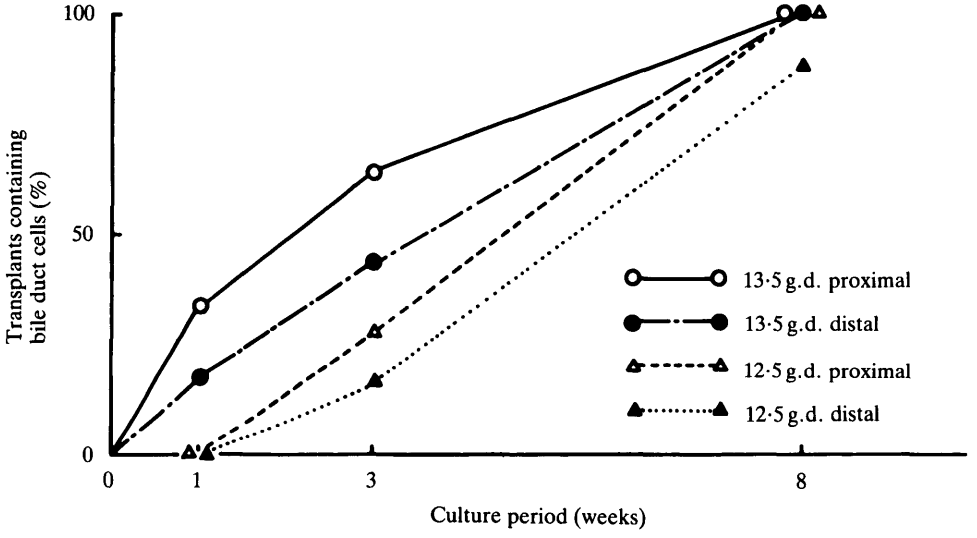


Fig. 7. Time course of the differentiation of bile duct cells in 12·5- and 13·5-day liver fragments cultured in the testis.

Table 1. *Histochemical characteristics of hepatocytes and bile duct cells in the normal adult liver, and 12·5- and 13·5-day liver fragments cultured in the testis*

	AFP	ALB	ALP	glycogen
hepatocytes in the normal adult liver	−	+	−	+
hepatocytes differentiated in the testis	−	+	−~+	+
intrahepatic bile duct cells in the normal adult liver	−	−~±	−	−
bile duct cells differentiated in the testis	−	−~±	−	−

AFP, alphafoetoprotein. ALB, albumin. ALP, alkaline phosphatase activity. −, not stained. ±, weakly stained. +, stained.

3. *Histochemical characteristics of large hepatocytes and bile duct cells differentiated in the testis*

Histochemical characteristics of mature hepatocytes and bile duct cells differentiated in the testis are summarized in Table 1.

Hepatocytes in liver fragments cultured for 8 weeks in the testis contained albumin, glycogen, but no detectable alphafoetoprotein, and often expressed bile canalicular alkaline phosphatase activity (Fig. 8). The characteristics of the hepatocytes differentiated in the testis were similar to those of adult hepatocytes. In contrast, differentiated bile duct cells were negative for alphafoetoprotein, alkaline phosphatase activity and glycogen, and negative or weakly positive for albumin (Fig. 8). These cells were also histochemically identical to the intrahepatic bile duct cells of the normal liver.

4. *Ultrastructure of large hepatocytes and intrahepatic bile duct cells differentiated in the testis*

Large hepatocytes differentiated in 13·5- and 18·5-day liver fragments cultured for 8 weeks in the testis possessed numerous round mitochondria, rough-surfaced endoplasmic reticulum, Golgi apparatus and glycogen granules. The plasma membrane of the hepatocytes displayed microvilli on the side of sinusoids, and Disse's space was formed. Bile canaliculi with microvilli were also seen between two hepatocytes (Fig. 9).

Bile duct cells differentiated in the testis were surrounded by a basal lamina on the basal surface, and possessed microvilli, and occasionally cilia, on the apical one. The bile duct cells joined each other by junctional complexes and interdigitations (Fig. 10). Golgi apparatus was situated in the supranuclear portion or in the lateral portion of the nucleus. Cell organelles developed poorly, and free ribosomes were abundant. The ultrastructure of the bile duct cells differentiated in the testis resembled that of intrahepatic bile duct cells.

5. *Differentiation of common bile duct cells in the testis*

To examine the possibility of the differentiation of hepatocytes from bile duct cells, common bile duct cells were transplanted into the testis. These cells differentiated into extrahepatic bile duct cells forming biliary glands, but did not differentiate into hepatocytes even after 3 weeks (0/7).

6. *Differentiation of intrahepatic bile duct cells from 13·5-day immature hepatocytes under the skin of newborn mice*

When 13·5-day liver fragments containing only immature hepatocytes as the endodermal component were transplanted under the skin of newborn mice and cultured for 1 week, most transplants were surrounded by dense connective tissue and developed bile ducts (Fig. 11). These bile ducts and small basophilic

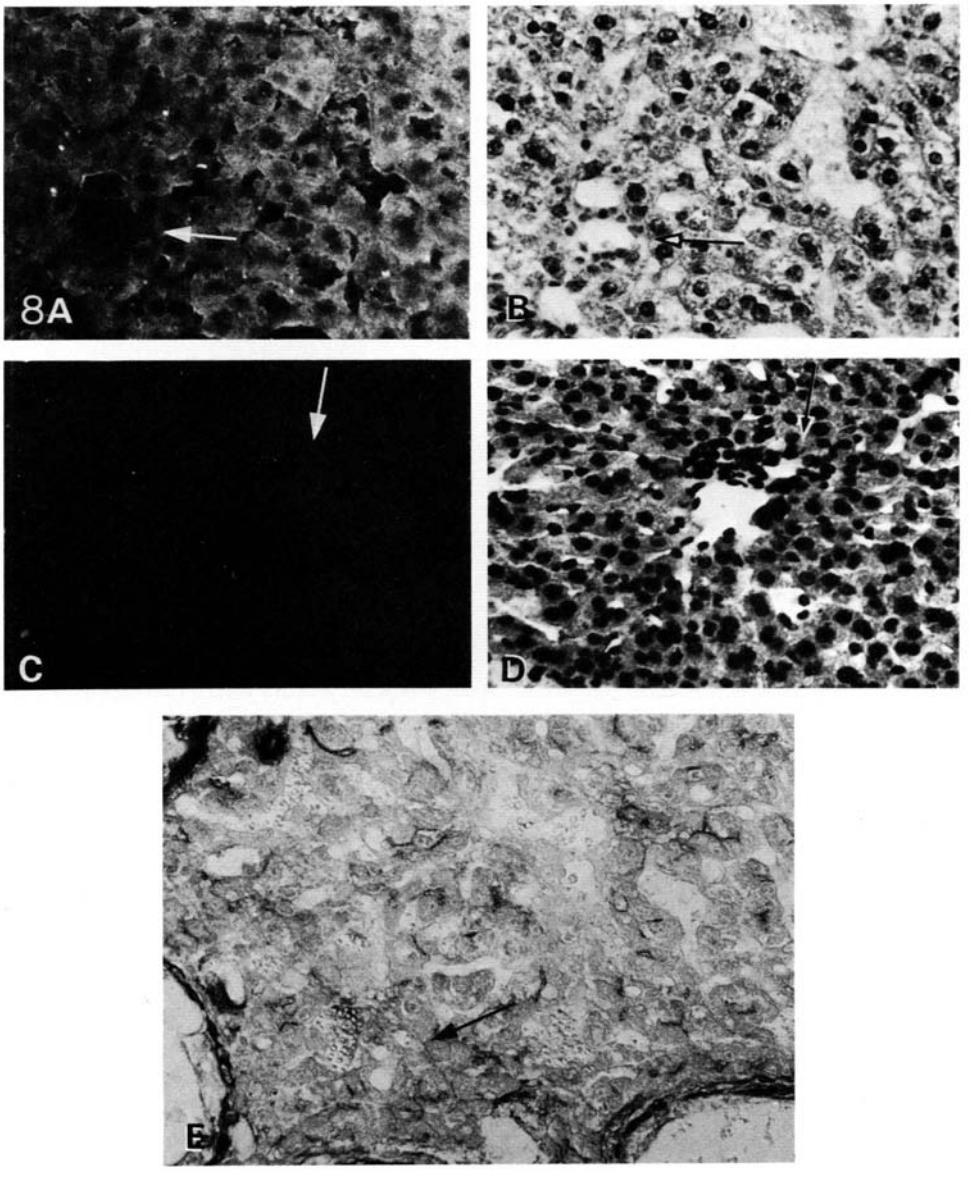
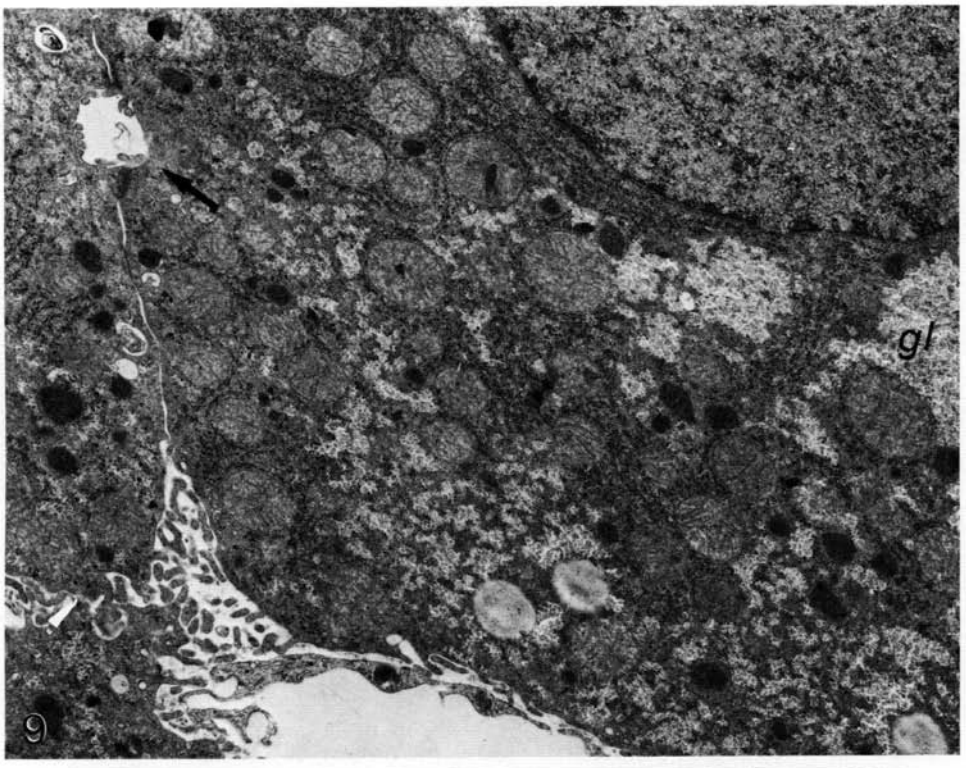
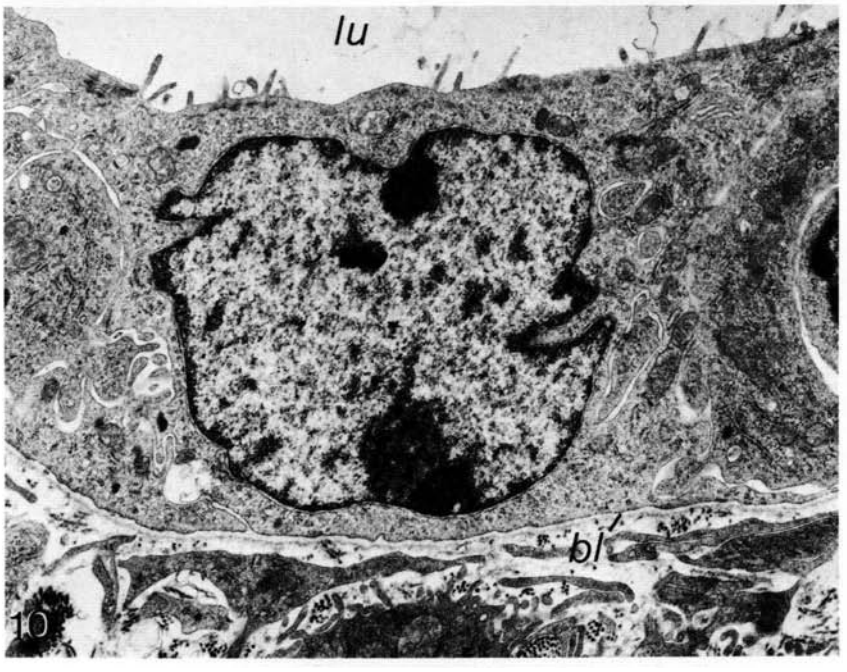


Fig. 8. A. Albumin immunofluorescence in a 13·5-day liver fragment transplanted into the testis and cultured for 8 weeks. Hepatocytes are positively stained for albumin, while intrahepatic bile duct cells (arrow) are negative. ×250. B. HX-E staining of A. ×250. C. Alphafoetoprotein immunofluorescence in a 13·5-day liver fragment transplanted into the testis and cultured for 8 weeks. Hepatocytes and intrahepatic bile duct cells (arrow) are negative. ×250. D. HX-E staining of C. ×250. E. Alkaline phosphatase activity in a 13·5-day liver fragment transplanted into the testis and cultured for 8 weeks. Bile canaliculi are positive, but intrahepatic bile duct cells (arrow) show no activity. ×250.





Figs 9 & 10

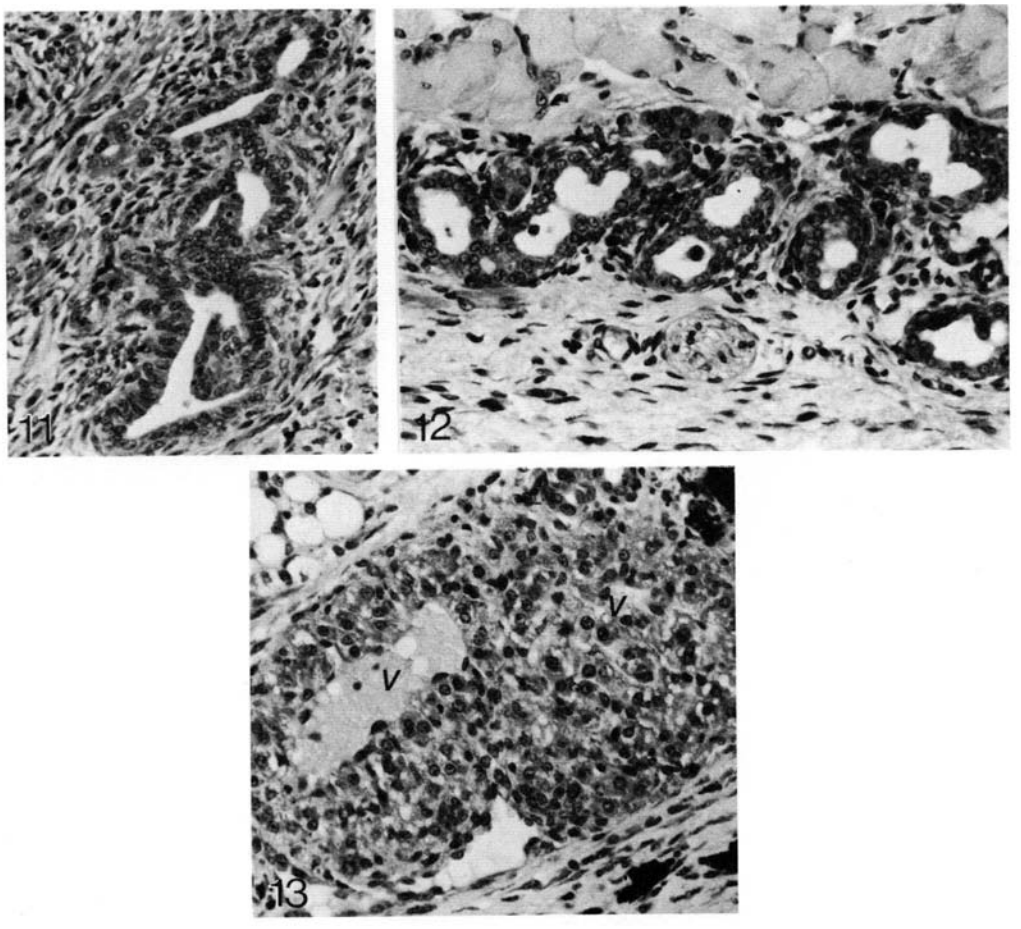


Fig. 11. A section of a liver fragment of a 13·5-day embryo transplanted under the skin of a newborn mouse and cultured for 1 week. The epithelial cells surrounded by the dense connective tissue form a tubular or glandular structure. PAS-HX. ×250.

Fig. 12. A section showing efficient differentiation of bile duct cells from 13·5-day immature hepatocytes under the skin of a newborn mouse. Three weeks' cultivation. PAS-HX. ×225.

Fig. 13. A section of a 13·5-day liver fragment containing no bile duct cells at the transplantation. Three weeks' cultivation under the skin of a newborn mouse. The hepatic tissue is constructed in the region invaded by blood vessels (v). PAS-HX. ×250.

Fig. 9. The ultrastructure of large hepatocytes differentiated in a 13·5-day liver fragment cultured for 8 weeks in the testis. Rough surfaced endoplasmic reticulum and round mitochondria are well developed. Arrow indicates a bile canaliculus. gl, glycogen. ×10 000.

Fig. 10. Bile duct cells in a 13·5-day liver fragment cultured for 8 weeks in the testis. bl, basal lamina. lu, lumen. ×10 000.

hepatocytes located at the periphery of the transplants. Especially in a region rich in the connective tissue, low columnar or columnar epithelial cells formed a tubular or glandular structure (Fig. 11). In contrast, large hepatocytes and many blood cells existed in the central region of transplants, especially when liver fragments were transplanted into the fat pad. Histologically, the bile duct formation in the transplants of 1 week's cultivation did not differ between each lobe, and between the proximal and distal liver fragments.

Bile duct cells differentiated efficiently in all subcutaneous transplants of 3 weeks' cultivation (63/63) (Fig. 12), and the proportion of the bile duct cells was about 60 % of endodermal epithelial cells in a subcutaneous transplant. The proportion of bile duct cells to the total endodermal epithelial cells was similar between the proximal and distal portions (results not shown). Biliary glands were formed only in the liver fragments containing the extrahepatic bile duct, and not formed in liver fragments containing only immature hepatocytes as an endodermal epithelium at the transplantation. However, large hepatocytes always differentiated, even in the subcutaneous cultivation which was more favourable to the bile duct formation. Most transplants were not rich in blood vessels and did not develop hepatocytes well, but the portion containing the vessels formed hepatic tissue rich in large hepatocytes (Fig. 13). All transplants of liver fragments containing the extrahepatic bile duct developed a hepatic architecture rich in sinusoids.

Even after 8 weeks of the transplantation, many bile ducts were maintained as well as those in 3 weeks' cultivation.

The bile duct formation in 13·5-day liver transplants was not influenced by the sex of hosts.

DISCUSSION

1. *The origin of endodermal epithelial cells in the mouse liver*

The origin of the endodermal epithelial cells in the mouse liver proposed in the present study is summarized in Fig. 14.

The present study showed experimentally that homogeneous immature hepatocytes can differentiate morphologically and functionally into mature hepatocytes and intrahepatic bile duct cells in ectopic sites. Especially when implanted under the skin, the immature hepatocytes could differentiate into the intrahepatic bile duct cells as rapidly as in the normal development of the liver. Therefore, it was found that the conditions under the skin are suitable for the differentiation of the immature hepatocytes into the intrahepatic bile duct cells, and their pathway of the differentiation is probable in the normal development of the mouse liver (Fig. 14). This view is consistent with the theory of Lewis (1911), Bloom (1926), Horstmann (1939), Elias (1955), Du Bois (1963), Wilson *et al.* (1963), Wood (1965), Picardi *et al.* (1968*a* and *b*) and Enzan *et al.* (1974).

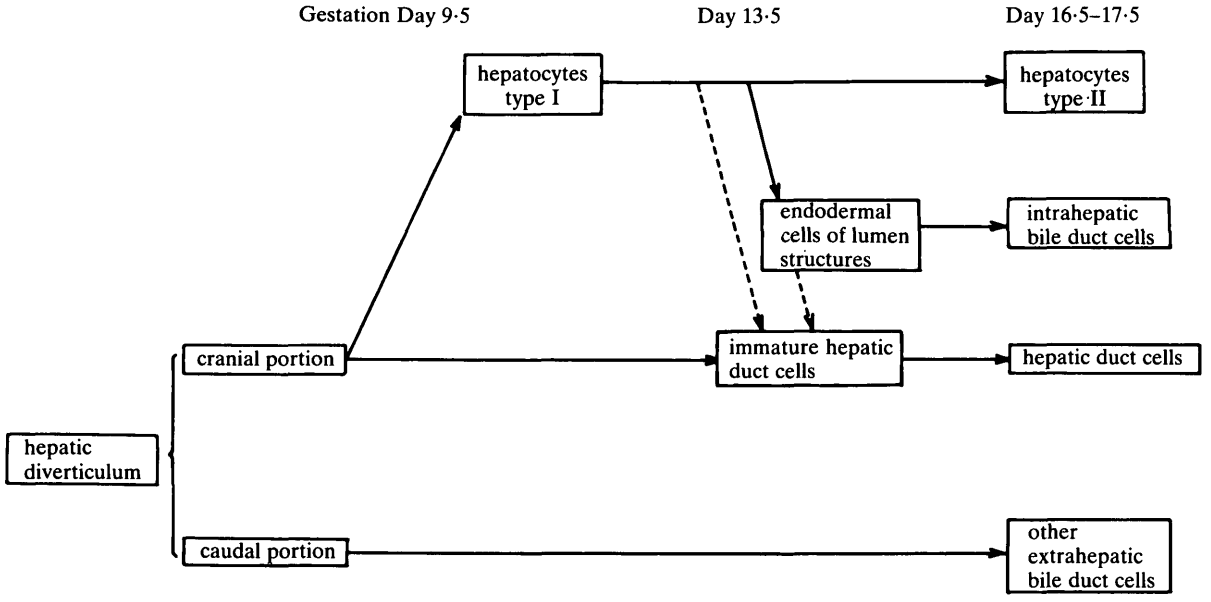


Fig. 14. The cell lineage of the endodermal epithelial cells in the ICR strain mouse liver. The development of the C3H/Tw strain mouse liver is about one day later than that of the ICR strain mouse liver. → means the possible differentiation pathway which could not be demonstrated experimentally. Hepatocytes type I mean immature hepatocytes, and hepatocytes type II mean large hepatocytes.

Although it could not be demonstrated in the present study that the hepatic duct cells forming biliary glands differentiate from the immature hepatocytes, at least a part of hepatic duct cells may originate from immature hepatocytes and endodermal cells of lumen structures (broken lines in Fig. 14) as suggested by Shiojiri & Mizuno (1983). However, the other part of the hepatic duct cells seems to originate directly from the endoderm of the cranial portion of the hepatic diverticulum (Fig. 14).

The present study showed that common bile duct cells failed to differentiate into hepatocytes. This suggests that extrahepatic bile duct cells do not differentiate into hepatocytes (Fig. 14).

2. *Differentiation of large hepatocytes and intrahepatic bile duct cells from immature hepatocytes*

In the present study, it was shown that the differentiation of the homogeneous immature hepatocytes into large hepatocytes and intrahepatic bile duct cells can be modified by experimental conditions. Large hepatocytes differentiated predominantly in testicular transplants and a part of subcutaneous transplant rich in vessels, and intrahepatic bile duct cells in subcutaneous transplant rich in the connective tissue. These results suggest that the contact of immature

hepatocytes with endothelial cells may be favourable to the differentiation of large mature-type hepatocytes, while the contact with the connective tissue cells may be favourable to the differentiation of bile duct cells.

3. *Polarity of the bile duct formation in the immature liver*

The bile duct differentiation which occurred in testicular transplants proceeded more rapidly in the proximal portion, though the differentiation of bile duct cells took place similarly between the subcutaneously transplanted proximal and distal liver fragments. These results suggest that 12·5- and 13·5-day livers polarized from the hilus to the periphery as to the differentiation of bile duct cells. The polarity could not be recognized under the subcutaneous conditions which are favourable to the differentiation of bile duct cells. Whether this polarity is characteristic of a population of immature hepatocytes, or due to the difference of distribution of other cell populations in the embryonic liver (e.g. haematopoietic cells, connective tissue cells, endothelial cells, etc.) is a problem to be resolved in the future. However, it has been shown that 12·5- and 13·5-day immature hepatocytes (11·5- and 12·5-day hepatocytes in the ICR strain mouse embryo) are homogeneous morphologically and histochemically (Shiojiri, 1979 and 1981). When the proximal and distal liver fragments in 12·5- and 13·5-day embryos were cultured in the testis for 8 weeks, bile ducts differentiated in most transplants. This result suggests that the immature hepatocytes possessing the competence to differentiate into the bile duct cells distribute throughout the liver parenchyma, but do not localize only in the hilus. That the polarity of the bile duct formation is due to the difference in the distribution of non-endodermal cells in the liver might be one of possibilities.

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